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To Scar or Not To Scar:
Origin and Function of Fibrotic Tissue in the Central Nervous System

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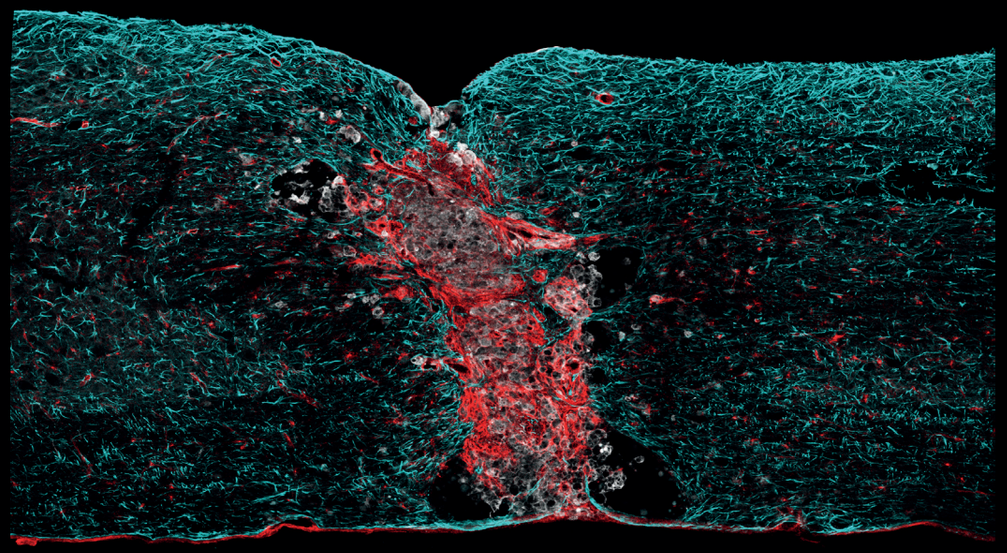

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TO SCAR OR NOT TO SCAR:

ORIGIN AND FUNCTION OF FIBROTIC TISSUE IN THE CENTRAL NERVOUS SYSTEM

David Oliveira Dias

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On the cover: The central nervous system scar, 14 days after a complete crush spinal cord injury in the mouse. Reactive glial cells marked by GFAP (cyan) form the glial component of the scar, and flank the non-neural, fibrotic lesion core composed of PDGFRβ-expressing stromal fibroblasts (red) and Mac2-positive inflammatory immune cells (white).

To Scar or Not To Scar: Origin and Function of Fibrotic Tissue in the Central Nervous System

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

David Oliveira Dias

The public defence of this thesis will take place in the lecture hall Biomedicum 1, Karolinska Institutet, Solnavägen 9, Solna, on Friday December 13 2019 at 9:30 am

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To my family and friends

‘Anything is possible if you’ve got enough nerve’

J. K. Rowling

ABSTRACT

After injury, the adult mammalian central nervous system lacks long-distance axon regeneration, and insufficient repair results in the formation of a multicellular and compartmentalized scar. A great body of work has been dedicated to the study of the glial component of the scar, while the fibrotic lesion core has received less attention. This thesis aims to deepen our knowledge on the cellular origin and function of fibrotic scar tissue following central nervous system injury and disease.

In **Paper I** we revealed that a subset of perivascular cells lining the vasculature, termed type A pericytes, is the major source of stromal fibroblasts that constitute the extracellular matrix-rich fibrotic component of the central nervous system scar following spinal cord injury in the mouse. Maximal genetic inhibition of proliferation by type A pericytes largely abolished fibrotic scar tissue generation and resulted in unsealed lesions and impaired wound healing, highlighting the importance of pericyte-derived scarring in regaining tissue integrity and wound closure. On the other hand, moderate inhibition of type A pericyte proliferation preserved wound closure and resulted in attenuated fibrotic scar tissue generation. This represented an attractive scenario to investigate the role of pericyte-derived scarring in axonal regeneration and functional recovery after spinal cord injury.

In **Paper II** we demonstrated that attenuation of pericyte-derived scarring is accompanied by decreased fibrosis, extracellular matrix deposition, astrogliosis and inflammation, and promoted regeneration of raphespinal and corticospinal tract axons caudal to the lesion. Corticospinal tract axons found below the injury site established functional synapses with local spinal neurons. Recovery of sensorimotor function was improved in animals with reduced pericyte-derived scarring. These results established pericyte-derived scarring as a therapeutic target to improve recovery following central nervous system injury.

In **Paper III** we asked whether generation of periycte-derived scar tissue is preserved across diverse central nervous system lesions. In addition to traumatic spinal cord injury, we found that type A pericyte progeny detached from the vascular wall and generated fibrotic scar tissue, or contributed to tumor stroma, after traumatic brain injury, inflammatory demyelinating disease and in a glioblastoma tumor model, respectively. Following cerebral ischemic stroke, type A pericytes increased in number but remained associated with the vasculature. We found that humans also develop fibrotic tissue enriched in stromal fibroblasts after central nervous system lesions, such as spinal cord injury and multiple sclerosis.

In **Paper IV** we showed that lesions to the spinal cord white matter trigger greater pericyte-derived fibrotic scarring compared to grey matter lesions. We demonstrated that myelin damage, myelin itself and myelin-associated proteins function as potent inducers of pericyte-derived fibrotic scarring, a process that is temporally synchronized with and dependent on the infiltration of peripherally derived macrophages. Reduction of monocyte-derived macrophage infiltration into the injured central nervous system or deletion of MAG, OMgp and Nogo, well-known myelin-associated axon growth inhibitors, resulted in attenuated fibrotic scar tissue generation following spinal cord injury.

The work presented in this thesis collectively supports a role for pericytes in fibrotic scar tissue formation and fibrosis following central nervous system injury. Interfering with pericyte-derived scarring may represent a promising therapeutic strategy to facilitate recovery following central nervous system injury and disease.

RESUMO

Após lesão, o sistema nervoso central de mamíferos adultos carece de regeneração axonal de longa distância, e a reparação insuficiente da lesão resulta na formação de uma cicatriz multicelular e compartimentalizada. Grande parte do trabalho de investigação desenvolvido tem-se dedicado ao estudo do componente glial da cicatriz, enquanto que o núcleo fibrótico da lesão tem sido menos estudado. Esta tese visa aprofundar o nosso conhecimento sobre a origem e função do tecido cicatricial fibrótico após lesão e em doenças do sistema nervoso central. No **artigo I**, revelámos que um subtipo de células perivasculares, denominado pericitos tipo A, é a principal fonte de fibroblastos do estroma que constituem o componente fibrótico, rico em matriz extracelular, da cicatriz do sistema nervoso central após lesão da medula-espinal do ratinho. A inibição genética máxima da proliferação dos pericitos do tipo A reduziu amplamente a formação de tecido cicatricial fibrótico e resultou em lesões não seladas e em deficiente cicatrização, salientando a importância das cicatrizes derivadas de pericitos na recuperação da integridade do tecido espinal e no fecho da lesão. Por outro lado, a inibição moderada da proliferação de pericitos do tipo A preservou o encerramento da ferida e resultou na formação atenuada de tecido cicatricial fibrótico. Este achado representou um cenário atraente para a investigação do papel das cicatrizes derivadas de pericitos na regeneração axonal e na recuperação de função após lesões da medula-espinal. No **artigo II**, demonstrámos que a atenuação de cicatrizes derivadas de pericitos é acompanhada por diminuição de fibrose, menor deposição de matriz extracelular e redução de astrogliose e inflamação, o que promoveu a regeneração dos axónios dos tractos rubro- e cortico-espinal abaixo do local da lesão. Os axónios do tracto cortico-espinal encontrados abaixo do local da lesão, estabeleceram sinapses funcionais com os neurónios espinais locais. A recuperação da função sensório-motora foi também melhorada em animais com cicatrizes menos densas em pericitos. Estes resultados estabeleceram que a cicatrização fibrótica derivada de pericitos é um alvo terapêutico para melhorar a recuperação após lesão do sistema nervoso central. No **artigo III**, investigámos se a formação de tecido cicatricial derivado de pericitos se encontra preservada em diversas lesões do sistema nervoso central. Além da lesão traumática da medula espinal, descobrimos que as células derivadas dos pericitos tipo A se dissociaram da parede vascular e geraram tecido cicatricial fibrótico, ou contribuiram para o estroma tumoral, após lesão cerebral traumática, doença desmielinizante inflamatória e num modelo de glioblastoma, respectivamente. Após acidente vascular cerebral isquémico, os pericitos do tipo A aumentaram em número, mas permaneceram associados à vasculatura. Comprovámos ainda que os seres humanos também desenvolvem tecido fibrótico enriquecido em fibroblastos após lesões do sistema nervoso central, nomeadamente lesão da medula-espinal e esclerose múltipla. No **artigo IV**, demonstrámos que lesões na matéria branca da espinal-medula geram maior cicatrização fibrótica derivada de pericitos em comparação com lesões na matéria cinzenta. Demonstrámos que danos causados à mielina, a própria mielina e as proteínas a esta associada funcionam como indutores potentes da cicatrização fibrótica derivada de pericitos, um processo que é sincronizado temporalmente e que depende da infiltração de macrófagos de origem periférica. A redução da infiltração de macrófagos derivados de monócitos no sistema nervoso central danificado ou a exclusão de MAG, OMgp e Nogo, conhecidos inibidores do crescimento de axónios associados à mielina, resultaram na formação atenuada de tecido cicatricial fibrótico após lesão medular.

O trabalho apresentado nesta tese suporta colectivamente um papel dos pericitos na formação de tecido cicatricial fibrótico e fibrose após lesão no sistema nervoso central. A manipulação das cicatrizes derivadas de pericitos pode, assim, representar uma estratégia terapêutica promissora para facilitar a recuperação após lesão ou em casos de doenças que afetam o sistema nervoso central.

LIST OF SCIENTIFIC PAPERS

I. Christian Göritz, **David O. Dias**, Nikolay Tomilin, Mariano Barbacid, Oleg Shupliakov and Jonas Frisén (2011). A Pericyte Origin of Spinal Cord Scar Tissue. *Science*, 333:238-242.

II. **David O. Dias**, Hoseok Kim, Daniel Holl, Beata W. Solnestam, Joakim Lundeberg, Marie Carlén, Christian Göritz† and Jonas Frisén† (2018). Reducing Pericyte-derived Scarring Promotes Recovery after Spinal Cord Injury. *Cell*, 173:153-165.

III. **David O. Dias***, Jannis Kalkitas*, Yildiz Kelahmetoglu*, Cynthia P. Estrada, Jemal Tatarishvili, Aurélie Ernst, Hagen B. Huttner, Zaal Kokaia, Olle Lindvall, Lou Brundin, Jonas Frisén and Christian Göritz. A Pericyte Origin of Fibrotic Scar Tissue Across Diverse Central Nervous System Lesions (*Manuscript*).

IV. Jannis Kalkitsas*, **David O. Dias***, Francesco Boato, Maria Kovatchka, Yutong Feng, Jian Zhong and Christian Göritz. The myelin components Nogo, OMgp and MAG induce fibrosis after CNS injury (*Manuscript*).

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Publications not included in this thesis:

Hanna Sabelström, Moa Stenudd, Pedro Réu, **David O. Dias**, Marta Elfineh, Sofia Zdunek, Peter Damberg, Christian Göritz and Jonas Frisén (2013). Resident neural stem cells restrict tissue damageand neuronal loss after spinal cord injury in mice. *Science*, 342:637-640.

Jens P. Magnusson*, Christian Göritz*, Jemal Tatarishvili, **David O. Dias**, Emma M. K. Smith, Olle Lindvall, Zaal Kokaia and Jonas Frisén (2014). A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. *Science*, 346:237-241.

David O. Dias and Christian Göritz (2018). Fibrotic scarring following lesions to the central nervous system. *Matrix Biology* 68-69:561-570.

* These authors contributed equally

CONTENTS

LIST OF ABBREVIATIONS

αSMA	Alpha smooth muscle actin
aa	Aminoacids
BAC	Bacterial artificial chromosome
BBB	Blood brain barrier
BPY-DCA	2,2'-Bipyridine-5,5'-decarboxylic acid
BSB	Blood spinal cord barrier
cAMP	Cyclic adenosine monophosphate
CCL2	C-C motif chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CD13	Alanyl aminopeptidase
CNS	Central nervous system
$CreER^{T2}$	Tamoxifen-inducible Cre recombinase
CSPG	Chondroitin sulfate proteoglycan
CST	Corticospinal tract
DSPG	Dermatan sulfate proteoglycan
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
HSPH	Heparan sulfate proteoglycan
iDTR	Inducible diphtheria toxin receptor
IFITM1	Interferon-induced transmembrane protein 1
IFN-γ	Interferon gamma
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
LPC	Lysophosphatidylcholine
LKZ	Leucine zipper-bearing kinase
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein

MCAO	Middle cerebral artery occlusion
MMC	Microvascular mural cell
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
MYL9	Myosin light chain 9
NG2	Chondroitin sulfate proteoglycan 4
NgR1	Nogo receptor 1
NVU	Neurovascular unit
OMgp	Oligodendrocyte-myelin glycoprotein
OPC	Oligodendrocyte precursor cell
PDGFRα	Platelet-derived growth factor alpha
PDGFRβ	Platelet-derived growth factor beta
PLP	Myelin proteolipid protein
PMP-22	Peripheral myelin protein-22
PNS	Peripheral nervous system
PTEN	Phosphatase and tensin homologue
RGS5	Regulator of G-protein signaling 5
RST	Raphespinal tract
SCI	Spinal cord injury
SMC	Smooth muscle cell
STAT3	Signal transducer and activator of transcription 3
SUR2	Sulfonylurea receptor 2
TBI	Traumatic brain injury
TGF-β	Transforming growth factor beta
TMEM119	Transmembrane protein 119
TNFα	Tumor necrosis factor alpha
YFP	Yellow fluorescent protein

1 INTRODUCTION

A healing response, involved in restricting tissue damage and restoring homeostasis, takes place after injury, and is followed by an attempt to restore tissue and organ function. Inflammation is coupled to active rebuilding and remodeling of the ECM, extensive cell proliferation and migration associated with dynamic restructuring of the tissue architecture, are conserved repair processes, although their success in restoring function varies across different tissues and species.

A scar, consisting of a multitude of resident and non-resident interacting cell types embedded in a complex ECM, forms as the result of attempted wound repair in mammalian organs. In various peripheral organs, the scar goes through a resolution phase and there is restoration of basic tissue functions, although not necessarily recapitulating the pre-injury state. Organs showing high regenerative capacity, such as the skin and the gastrointestinal tract, recruit resident stem cells to sustain tissue function. Whereas, organs presenting lower cellular turnover, such as the liver and lungs, mainly depend on proliferation of committed progenitor pools (*1*).

In the CNS, tissue scarring exhibits a higher degree of complexity. It is commonly accompanied by reactive tissue changes such as inflammation, altered deposition of ECM components, fibrotic scarring and reactive gliosis (*2–4*). Injury-induced remodeling programs fail to reestablish tissue organization and pathology remains chronically, compromising tissue functions within and surrounding the non-resolving scar tissue (*2*). It has been increasingly recognized over the years that scar tissue, as a healing response, is highly necessary to contain the damage and limit further injury spread. However, the cellular and extracellular components of the scar microenvironment do also play key roles in limiting functional repair and contribute to the failure of axon regeneration after CNS injury (*3*, *5*).

Among several other CNS pathologies that exhibit reactive tissue changes and scarring, wound repair is particularly inefficient and pathological changes persist chronically after traumatic SCI. Increased levels of infiltrating leukocytes with a capacity to exacerbate tissue damage and enhanced expression of inflammatory cytokines, associated with remarkable astrocyte activation and ECM deposition, may underlie the limited capacity for repair of the injured spinal cord compared to brain injuries (*2–4*, *6–9*).

2 TISSUE SCARRING IN THE INJURED CNS

2.1 CASCADE OF CELLULAR EVENTS FOLLOWING TRAUMATIC AND ISCHEMIC INSULTS

Acute focal injuries in the CNS, including traumatic injury and ischemic stroke, trigger wound repair with tissue replacement. A complex multicellular response is elicited, in which CNS intrinsic neural-lineage cells (neurons, oligodendrocytes, OPCs, astrocytes and ependymal cells) interact with non-neural resident cells, including microglia, endothelial cells, meningeal fibroblasts, pericytes and other perivascular cells (*3*, *5*). Peripheral myelinating Schwann cells can also migrate and invade the CNS after injury (*10*). Additionally, monocyte-derived macrophages, neutrophils and lymphocytes (T cells, B cells and NK cells) as well as other bone marrow-derived cells such as fibrocytes and platelets are recruited from the circulation and infiltrate the injured CNS (*5*). Acute focal traumas caused by contusion or crush are accompanied by pronounced vascular damage and therefore, present many similarities to ischemic injury, such as cerebral ischemic stroke.

CNS responses to acute focal injury trigger a multitude of sequential and overlapping events that can be split into three main phases (*5*), summarized in **Figure 1**.

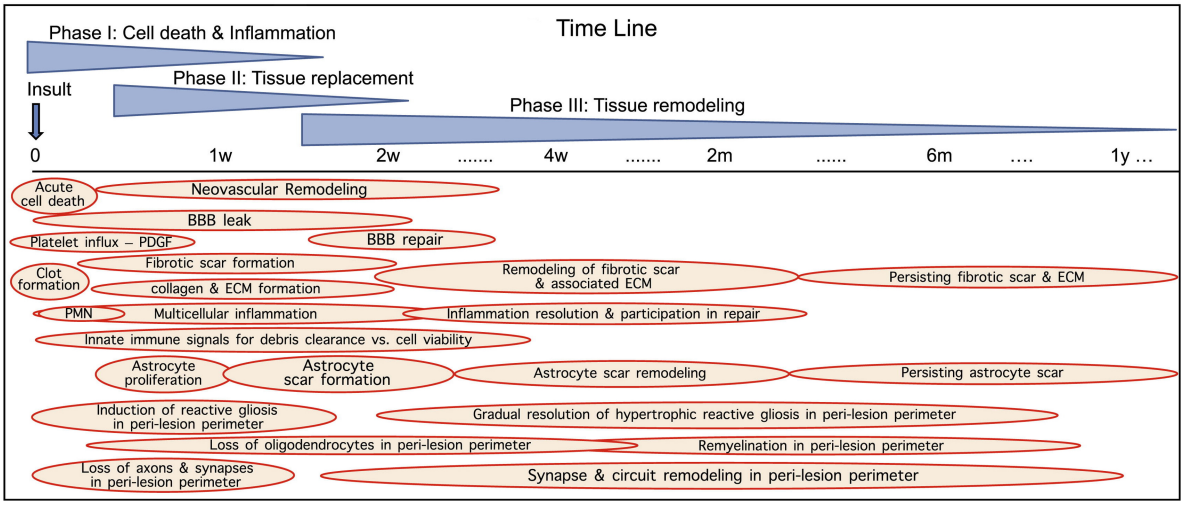

Figure 1 | Phases and time course of multicellular responses following acute focal CNS insults
Traumatic and ischemic injuries to the CNS trigger a complex cascade of overlapping but distinct events, that include cell death and inflammation (Phase I), cell proliferation and replacement (Phase II), and tissue remodeling (Phase III), and involve the crosstalk among neural and non-neural cells intrinsic to CNS and non-neural cells infiltrating from the circulation
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2.1.1 Phase I - Cell death and inflammation

Seconds to hours after the primary insult and subsequent events that gradually develop over days

A traumatic or ischemic insult leads to acute death of local cells and axonal damage, disruption of the vasculature and loss of integrity of the BBB/BSB. Endothelial cell trauma rapidly triggers platelet adhesion and activation, the coagulation cascade and blood clot formation at the injury core (*5*, *11*, *12*). The initial damage initiates a secondary injury cascade characterized by haemorrhage, local oedema and swelling, release of cytotoxic products and cell death, in particular neurons and oligodendrocytes (*11*). Microglia promptly sense and migrate towards the site of tissue damage to protect against spread of damage, initiate the clearance and removal of debris and stimulate the recruitment of other cells (*13–15*). Neutrophils, lymphocytes and peripherally-derived macrophages infiltrate the injured CNS to scout for pathogens, phagocytose debris and instruct the wound repair process (*16*, *17*). In contrast to OPCs that show chemotaxis towards the injury zone (*18*, *19*), astrocytes do not migrate but become reactive and hypertrophic, and occasionally proliferate. Following severe trauma or ischemia, astrocytes in the lesion center can die (*20–23*). Tissue reperfusion potentiates oxidative stress and glutamate excitotoxicity-mediated death of neighboring neurons and glia, and further axonal damage (*24*). The sustained secondary injury cascade exacerbates and often surpasses the damage caused by the initial insult.

2.1.2 Phase II - Cell proliferation for tissue replacement

2 to 10 days after the primary insult

The proliferative and tissue replacement phase that takes place in response to acute CNS tissue damage resembles in many aspects classic wound healing responses in other tissues (*25–27*). It is characterized by prominent proliferation of endothelial cells and progenitors, involved in neovascularization of the injury site via sprouting angiogenesis (*28*, *29*). Additionally, widespread deposition of ECM molecules, proliferation and migration of inflammatory cells, fibroblast-lineage cells and pericyte-derived cells (**Paper I**) contribute to tissue replacement by forming fibrotic scar tissue (*30–33*). Although angiogenesis takes place, the integrity of the BBB/BSB in the lesion core is not reestablished yet and the leaky vessels allow free extravasation of serum proteins and other molecules into the neighboring neural parenchyma (*34*). Additionally, there is extensive proliferation of CNS intrinsic neural cells, including local astrocytes, scar-participating OPCs and ependyma-derived cells that later assemble and participate in the formation of a mature CNS scar (*19*, *35–45*). Interestingly, striatal parenchymal astrocytes are able to enter a neurogenic program and generate neuroblasts that later mature into neurons after cerebral ischemic stroke, a process

that depends on Notch signaling (*18*, *46*, *47*). Likewise, forebrain ependymal cells were shown to produce neuroblasts and astrocytes in response to ischemic stroke, via a Notch dependent mechanism (*48*). Moreover, in response to acute CNS damage in the forebrain, subventricular zone-lining neural stem cells generate progeny that migrate towards the injured cortex and striatum and contribute to newly generated neurons and glial cells in perilesion perimeters (*49–52*).

2.1.3 Phase III - Tissue remodeling

From the end of the first week after the primary insult

The tissue remodeling phase is marked by the organization of injury-participating cells as well as extracellular components that assemble to form the CNS scar, and by the restoration of the BBB/BSB and neurovascular unit around newly formed blood vessels of the lesion penumbra (*33*, *34*, *53*).
Reactive astrocytes become hypertrophic, upregulate the expression of intermediate filaments such as nestin, vimentin and GFAP and extend well-organized interdigitating processes (*21*, *54–57*) that form a barrier-like structure (*58*) and flank a fibrotic lesion core of non-neural tissue by 2 to 3 weeks after an acute CNS insult (*45*). As axons continue to degenerate and retract away from the lesion center, oligodendrocyte death continues, triggering additional local tissue responses. With time, remodeling of the lesioned tissue continues with gradual contraction of glial and fibrotic scar components, and ongoing alterations in the composition of the ECM (*32*, *59–61*). As acute inflammatory responses taper off, the spared but reactive neural tissue surrounding mature lesions undergoes a gradual and continuous remodeling of neural circuits and attempt to remyelinate denuded axons (*2*, *12*).
In humans and rats, thin bands of fibrous tissue and macrophages develop around cysts after some types of traumatic SCI and further restrict axonal regrowth and cell migration. (*3*, *62–67*).

2.2 SCAR FORMATION AFTER TRAUMATIC SCI

The lesion environment is a complex mixture of interacting cell types that react to injury in a stereotyped fashion, forming a mature scar (*2*, *3*, *5*, *68*). Over the last decades, astrocytes, NG2-expressing OPCs, ependymal cells, peripherally-derived macrophages, microglia, meningeal cells, pericytes and other perivascular cells have been investigated in the context of scar formation after CNS trauma, namely SCI (*2*, *3*, *5*, *68*).
The mature CNS scar that forms after traumatic SCI, erroneously referred to as the glial scar as a whole, can be grossly viewed as a two-compartment structure, where a reactive glial scar

border intimately flanks a central lesion core filled with non-neural fibrotic tissue, often referred to as fibrotic scar (**Figure 2**). Additionally, a large perilesion area of functional but reactive neural tissue exhibiting attenuating reactive gliosis that gradually transitions to healthy tissue can be appreciated and was recently proposed as a third compartment of the chronic matured scar (*3*, *5*). Despite becoming spatially segregated in a chronic scar, there is temporal dependence and bidirectional cross talk amongst scar components.

2.2.1 Glial scar

Severe tissue damage accompanied by BBB/BSB breakdown, extravasation of serum proteins into the CNS parenchyma and leukocyte infiltration, trigger rapid migration of astrocytes away from the inflammatory epicenter (*2*). These processes initiate the formation of a glial, or astrocyte, scar at the lesion penumbra, characterized by astrocyte hypertrophy. Hypertrophic astrocytes undergo massive restructuring that leads to the formation of compact astrocyte scars, a specialized aspect of reactive astrogliosis, which depends on IL-6 receptor-STAT3 and LKZ signaling (*35*, *45*, *57*).

Glial scar formation around central lesion cores is largely completed by 2–4 weeks after traumatic SCI, a time when the lesion is considered mature and enters its chronic stage. The compact glial scar border serves to directly demarcate and contain areas of fibrotic non-functional lesion core tissue from the immediately surrounding viable but reactive neural tissue. It is composed of densely packed astrocytes with elongated processes that intermingle and intertwine extensively and some NG2-expressing OPCs. Spinal cord astrocytes and central canal linining-ependymal cells are the two main cellular sources of newly proliferated scar-forming astrocytes after SCI in rodents (*38*, *42*, *45*, *69*). In response to trauma, NG2-expressing OPCs proliferate and migrate towards the injury site. Apart from their known role in generating remyelinating oligodendrocytes, either by oligodendrogenesis or via differentiation into remyelinating Schwann cells (*10*, *70–73*), NG2-expressing scar-participating OPCs hyperthrophy, upregulate NG2, accumulate within the glial scar and some differentiate into astrocytes, after traumatic SCI (*18*, *36–38*, *74*, *75*).

In contrast to peripherally-derived macrophages that associate with fibroblast-lineage cells in the lesion core, microglia proliferate and accumulate at the interface between fibrotic and astroglial scars after SCI (*14*, *76–78*).

2.2.2 Fibrotic scar

The wound healing response triggered by CNS injury recruits local and infiltrating immune cells, as well as ECM-producing stromal fibroblasts. Although some tissue remodeling is

appreciated over time, these cells do not withdraw entirely and are not replaced by regenerated tissue, persisting chronically in the lesion core (*79*).

The mature CNS scar presents a central lesion core, often referred to as the fibrotic scar (alternative nomenclature includes: mesenchymal, fibrous, collagenous matrix or connective tissue scar), composed of non-neural tissue, and immediately surrounded by the glial component of the scar (*3*, *79*). Major components of this non-neural tissue are endothelial cells and progenitors, inflammatory immune cells, including monocyte-derived macrophages, stromal fibroblasts, also referred to as fibroblast-related cells or fibroblast-like cells, and ECM deposits (*3*, *80*). Endothelial cells, stromal fibroblasts and monocyte-derived macrophages contribute to the excessive deposition of connective tissue matrix. In addition to numerous others ECM components, the lesion core contains glycoproteins such as CSPG and HSPG, and collagens, fibronectin and laminin (*32*, *60*, *61*, *81–84*). The prolonged presence of these matrix molecules delays tissue remodeling by fueling fibrosis and scarring via interaction with inflammatory cells (*2*, *85*).

The origin of stromal fibroblasts residing in fibrotic scar tissue after CNS injury and disease was investigated in **Papers I** and **III**.

2.2.3 Interaction of cellular scar components

Due to their strategic location at the glial-fibrotic interface of the scar, reactive astrocytes can interact and be influenced by stromal fibroblasts and inflammatory cells (*45*). After CNS injury, reactive CNS astrocytes expressing the ephrin-B2 ligand intermingle with stromal fibroblasts expressing the EphB2 receptor. This interaction culminates with the formation of restricted cellular domains containing dense networks of cells and interweaving processes and the establishment of an astroglial-fibrotic lesion border in the sub-acute phase after SCI (*86*). More recently, type-I collagen, expressed by stromal fibroblasts within the lesion core, was found to induce astrocytic scar formation via the integrin–N-cadherin pathway (*87*). In another recent study, perivascular PDGFRβ-expressing cells (described as reactive pericytes) were found to upregulate the expression of the ECM molecule periostin after SCI and mediate the upregulation of TNFα from infiltrating monocyte/macrophages to promote scar formation, type-I collagen deposition and fibrosis after SCI (*88*).

Perivascular astrocyte endfeet lie in a prime location and intimately border the basal lamina of endothelial cells at the BBB/BSB, making astrocytes pivotal regulators of resident and infiltrating immune cells during CNS scar formation (*89*). Indeed, the release of CCL2, IL-1β, IL-6 and other chemokines and cytokines by reactive astrocytes triggers polarization of microglia and peripherally-derived macrophages into a pro-inflammatory phenotype. The release of inflammatory mediators such as IFN-γ, IL-6, IL-1β and TNFα by immune cells

triggers astrocyte reactivity and astrogliosis (*21*). Interestingly, activated neuroinflammatory microglia are capable of inducing a neurotoxic phenotype in reactive astrocytes, via the release of Il-1α, TNF and C1q (*21*, *90*). Additionally, microglia/macrophages express the ECM-associated protein osteopontin and promote glial scar formation after ischemic brain injury via binding to $\alpha_v\beta3$ integrin on reactive astrocytes (*91*).

Apart from direct cellular interactions, parenchymal cells can sense signals from the injury milieu and indirectly influence the behavior of neighboring cells (*2*).

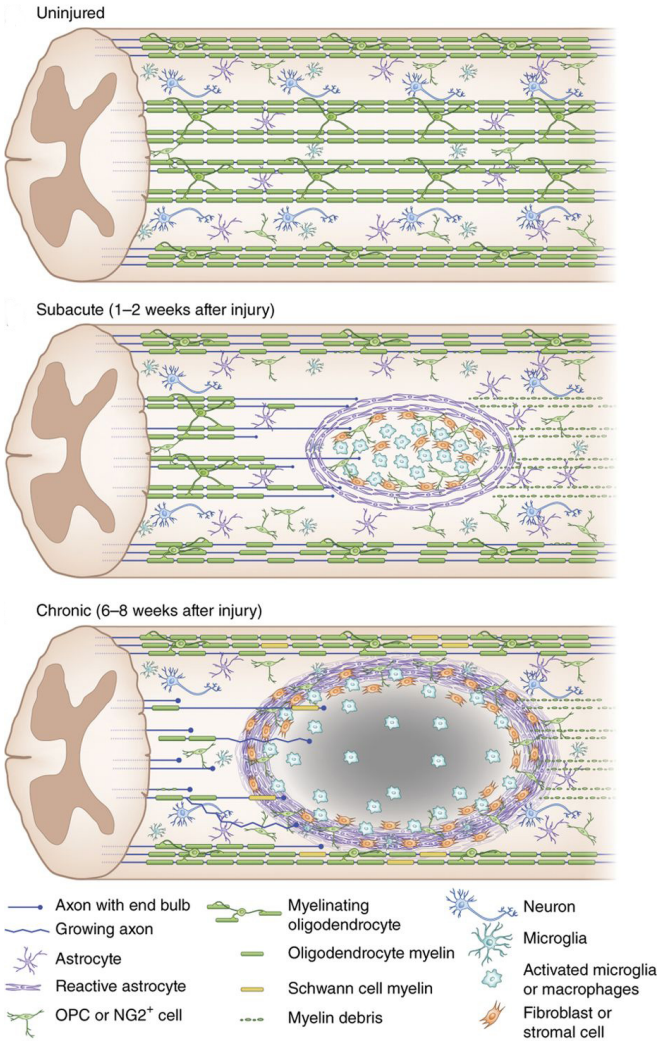

Figure 2 | Scar formation after traumatic SCI
Schematic representation of the uninjured, and injured spinal cord at subacute and chronic stages after a contusive type lesion in the rat. SCI triggers a complex multicellular response, involving CNS resident and non-resident cell types, which culminates with the formation of a scar at subacute stages, that persists chronically. In the subacute stage, there is substantial loss of neurons, axons, oligodendrocytes and myelin. Axons retract away from the lesion epicenter. Activated microglia, infiltrating monocyte-derived macrophages, among other inflammatory immune cells, are recruited to the injury site and assist in the clearance of myelin debris. The CNS scar exhibits a central fibrotic component, packed with inflammatory cells and stromal fibroblasts, surrounded by an outer glial component, made of reactive astrocytes and some NG2-expressing OPCs.
In the chronic phase, OPCs and Schwann cells contribute to generation of newly myelinating oligodendrocytes. Scar maturation is accompanied by a dampened, but persisting, inflammatory response, and cavitation, in the rat after contusion SCI. Fibrotic and glial scar components remain chronically. Reprinted by permission from Springer Nature (*92*), © 2017.

2.2.4 Dual role of scar components

Increasing evidence supports a dual, and seemingly opposing, role of the CNS scar, in both promoting tissue protection but also inhibiting repair.
Scar-forming astrocytes have been extensively studied and regarded as one of the main sources of axon growth inhibitory CSPGs (*93–99*). However, the notion that astrocyte scars

act as a physical and molecular barrier that hinders rather than aids axon regeneration was recently challenged (*87*, *100*). A potential source of discrepancy may arise from the heterogeneous origin of scar forming-astrocytes (*38*, *42*). Selectively blocking the generation of ependymal cell-derived scar-forming astrocytes after SCI revealed their importance in restricting secondary tissue damage and mediating tissue protection and, therefore, supporting regeneration (*40*, *41*). This is in agreement with other studies that recognize the vital role of reactive astrocytes in bordering the fibrotic lesion core and shielding nearby viable neural tissue from destructive inflammation (*34*, *35*, *43*, *45*, *55*, *57*, *100–104*). It has also been documented that adult mammalian CNS astrocytes form permissive bridges *in vivo*, known as glial bridges, along which injured CNS axons can regrow and cross the scar when stimulated with appropriate growth factors, grafts, and by genetic activation of neuronal intrinsic growth programs (*100*, *105–108*).

Activated macrophages and microglia dominate sites of CNS injury and play crucial roles in promoting tissue remodeling and repair by clearance of cellular and myelin debris, degradation of scar tissue and production of growth factors, but can also drive secondary injury through a vicious neurotoxic inflammatory cycle (*109*). Depending on the microenvironment and route of entry into the injured CNS, macrophages can polarize into a classically activated pro-inflammatory and detrimental phenotype (named M1) that can cause neurotoxicity, or an alternative, anti-inflammatory pro-repair phenotype (named M2) (*78*, *110*, *111*). Given the pro-inflammatory milieu within the injured spinal cord, there is a predominance of an M1 macrophage response that perpetuates and is only counteracted by a smaller and temporary M2 macrophage response. Although of common origin, monocyte-derived M1 macrophages infiltrate the CNS via the adjoining spinal leptomeninges, while monocyte-derived M2 macrophages enter from the brain-ventricular choroid plexus of the blood-cerebrospinal fluid barrier (*111*). A similar pattern of polarization is observed in microglia following injury (*112*). M1 pro-inflammatory, neurotoxic microglia posses low phagocytic activity and are involved in secondary tissue damage and glial scar formation after SCI (*113*, *114*). In turn, M2 microglia possess increased phagocytic activity, produce growth factors and anti-inflammatory cytokines and thereby, stimulate tissue repair and regeneration (*112*).

Activated microglia were recently shown to promote tissue repair and constitute an essential component of the neuroprotective scar that forms after SCI. The elimination of microglia in the subacute phase after traumatic SCI leads to reduced astrocyte proliferation and disruption of the glial scar, and increased infiltration of monocyte-derived macrophages, resulting in increased death of oligodendrocytes and neurons, and impairing functional recovery (*14*, *77*) . In addition to its well-known activity in limiting axonal growth and representing a major obstacle for CNS recovery after injury, CSPG deposition was shown to play a key role in controlling the secretion of neurotrophic factors by resident microglia and infiltrating monocyte-derived macrophages, thereby promoting repair and functional motor recovery during the acute phase after traumatic SCI, but not at later stages (*115*, *116*).

Following CNS injury, NG2-expressing OPCs were shown to migrate towards the lesion site and participate in the formation of the glial scar, a structure that, as discussed above, exerts neuroprotective effects by limiting the spread of neurotoxic inflammatory lesion core cells. Interestingly, NG2 glia was also found to entrap dystrophic axons by forming synaptic-like connections (*117*) and stabilize dystrophic axons undergoing pro-inflammatory macrophage-mediated axonal dieback after SCI (*118–120*).

In **Papers I** and **II**, we will present evidence for a dual role of pericyte-derived scar tissue after SCI. On the one hand, scar-forming pericyte-derived cells are essential for sealing off the lesion site and the reestablishment of tissue integrity. On the other hand, pericyte-derived fibrotic scarring inhibits axon regeneration and limits functional recovery following SCI (*30*, *79*).

3 STROMAL FIBROBLASTS - CELL OF ORIGIN

Scar formation has been reported in humans and experimental animal models in response to traumatic lesions to the brain and spinal cord, stroke, autoimmune demyelinating lesions, such as MS, and tumors (*5*, *6*, *102*, *121–131*).

The glial component of the CNS scar that forms in adult mammals has been extensively studied and is mainly composed of reactive astrocytes (*36*, *38*, *94*). Although acknowledged, the fibrotic component of the scar, mostly comprised of inflammatory immune cells and stromal fibroblasts, has received less attention. In particular, the cellular origin of stromal fibroblasts that form fibrotic scar tissue has been difficult to establish.

Fibroblasts are versatile tissue-resident mesenchymal cells found in the interstitial space of organs. These cells are typically spindle-shaped and present an oval flat nucleus. Fibroblasts can produce or respond to a wide range of cytokines and their primary function is to synthetize, secrete ECM proteins, including collagens, tenascin, laminin, fibronectin and proteoglycans (*132*). Together with endothelial cells and macrophages, activated fibroblasts are critically involved in the remodeling phase of wound healing. Under conditions in which successful wound healing occurs, fibroblasts differentiate into αSMA-expressing myofibroblasts that can produce and secrete ECM and actively participate in the wound closure process by promoting tissue contraction (*132*). As tissue remodeling progresses and adequate ECM has been deposited, fibroblasts and myofibroblasts gradually disappear, thereby limiting disproportionate ECM secretion and dampening the pro-inflammatory and pro-fibrotic environment (*132*).

Due to the ongoing persistent inflammation (*133*, *134*), these cells are not cleared off from CNS scar tissue and, together with inflammatory cells, cluster at the lesion core and remain indefinitely.
In the CNS, several cell types have been proposed to contribute to scar-forming stromal fibroblasts and are discussed below.

3.1 SPINAL CORD INJURY

Following traumatic injuries to the mouse spinal cord, including dorsal hemisection, contusion and crush spinal lesions, fibrotic scar tissue accumulates at the lesion core surrounded by a glial scar (**Figure 3A**,**E**) (*30*, *31*, *106*, *107*, *135*, *136*). Rats and humans, but not mice, show cavitation at the lesion site after contusive spinal injuries (*62*, *66*, *137*). Interestingly, fibrotic scarring still occurs in these contusion injuries containing multiple hemorrhagic and necrotic regions (*3*, *31*, *62*, *76*, *135*, *137–139*). Additionally, generation of

fibrous scar tissue enriched with inflammatory cells and excessive ECM deposits is observed after crush, compression and lacerating spinal injuries in humans (*3*, *62*, *137*, *139*, *140*). Spinal crush in rodents is, therefore, a clinically relevant model of human SCI that occurs as a result of vertebral crush or displacement (*106*, *107*, *136*, *141*). In cases the *dura mater* is breached, meningeal fibroblasts can infiltrate the lesion site and contribute to fibrotic tissue generation (*86*, *142–144*).

Collectively, different scar-participating cell types, including stromal fibroblasts, meningeal fibroblasts, monocyte-derived macrophages, astrocytes, and endothelial cells, contribute to the deposition of excessive amounts of fibrillar collagens (types I, III, and V), fibronectin, tenascin-C, periostin, in addition to basal lamina components, such as laminins and collagen type IV, and mediate tissue fibrosis in SCI (*3*, *59*, *88*, *135*, *138*, *143*, *145–149*).
Until our work (**Papers I** and **III**), CNS-resident perivascular fibroblasts (*31*, *76*) or meningeal-derived fibroblasts have been suggested as the primary source of scar-forming stromal fibroblasts after SCI (*32*, *84*, *144*, *150–152*).

3.2 TRAUMATIC BRAIN INJURY

Brain trauma is accompanied by disruption of the BBB, acute pericyte loss and accumulation of inflammatory cells into the lesion area, followed by scar formation (*143*, *153*). A fibrotic lesion core enriched in PDGFRβ-expressing stromal fibroblasts and fenced off by a glial scar develops after penetrating and non-penetrating injuries to the brain, such as stab wounds (**Figure 3B**,**F**) and cortical controlled impact and lateral fluid percussion-induced injuries, respectively (*143*, *153*).
Pericytes were shown to detach from the vascular wall within the first hour after TBI (*154*). Pericyte-derived cells and fibroblasts invading from the nearby meninges have been suggested to deposit ECM, including collagen type IV, fibronectin and laminin, and generate fibrotic tissue after brain injury (*81*, *143*, *153*, *155–160*).

3.3 STROKE

After cerebral ischemic stroke, a glial scar develops and surrounds an ischemic lesion core undergoing extensive vascular remodeling in both humans and rodents (*161–163*) (**Figure 3C**,**G**). Increased expression of PDGFRβ, fibronectin, collagen type 1 and periostin was reported within the ischemic stroke core and in perilesion areas (*164–171*).
A dense network of PDGFRβ-expressing cells, presumably derived from the neurovascular unit, was suggested to deposit fibrous ECM and contribute to post-ischemic scarring (*121*, *131*, *171*). In addition, a meningeal-derived population of Col1a1-expressing perivascular

stromal cells was suggested to generate fibrotic scar tissue after experimental cerebral ischemia (*172*). Bone marrow-derived cells do not appear to participate in fibrotic scarring in the post-ischemic brain (*121*, *131*).

3.4 MULTIPLES SCLEROSIS

MS is an autoimmune demyelinating disease of the CNS characterized by the presence of multiple white matter scars across the brain and spinal cord. Demyelination with axonal damage, glial scar formation and perivascular aggregates of PDGFRβ–expressing cells intermixed with monocyte-derived macrophages and lymphocytes, are detected near post-capillary venules following EAE in the mouse (**Figure 3D**,**H**) and in active MS brain lesions (*128–130*).

Extensive deposition of fiber-like ECM within inflammatory perivascular cuffs, including accumulation of fibrillar collagens, fibronectin, agrin, biglycan and decorin, is apparent in both active MS lesions and following EAE (*126*, *128*, *130*, *173–175*). In addition, perivascular and parenchymal expression of tenascin-C and -R proteins, laminins, CSPGs, HSPGs, and DSPGs is altered within demyelinated areas.

3.5 BRAIN TUMORS

Reactive gliosis and the presence of a tumor stroma enriched in ECM, vasculature, inflammatory immune cells and stromal fibroblasts are characteristics of brain tumors, such as glioma (*124*).

Collectively, all the aforementioned studies acknowledge the presence of fibroblasts in fibrotic tissue following CNS injury and disease. However, no genetic lineage tracing strategies have been employed in these studies to unequivocally sort out the cellular origin of scar-forming stromal fibroblasts.

The cellular origin of stromal fibroblasts that form fibrotic scar tissue following penetrating and non-penetrating SCI, TBI, MCAO-induced ischemic stroke, EAE and in a glioblastoma tumor model was subjected to investigation in **Papers I** and **III**.

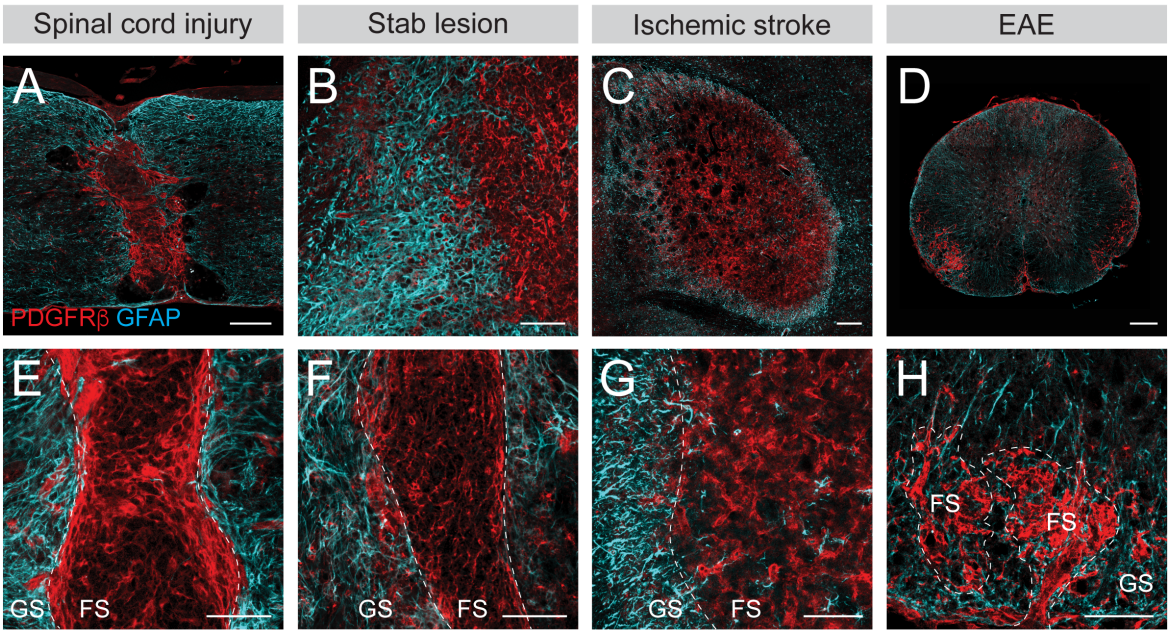

Figure 3 | Scar formation following lesions to the adult mammalian brain and spinal cord Fibrotic scar (FS; PDGFRβ) and glial scar (GS; GFAP) components following complete crush SCI (**A**,**E**), cortico-striatal stab lesion (**B**,**F**), MCAO-induced ischemic injury (**C**,**G**) and EAE (**D**,**H**).
A,E and B show sagittal views of the injured spinal cord and brain, respectively; C,F,G and D,H show coronal views of the brain and spinal cord, respectively. Scale bars represent 100 μm (B,E-H) and 200 μm (A,C,D). Reproduced from (*79*).

3.6 MANIPULATION OF FIBROTIC SCAR TISSUE TO PROMOTE AXON REGENERATION

When a CNS axon is transected, the distal segment undergoes Wallerian degeneration. However, the proximal portion of the axon initially retracts away from the original site of injury, a process known as axonal dieback, but its neuronal cell body can subsist for years after axotomy (*176–178*). Some severed axons initiate a growth response but ultimately fail to cross beyond the lesion (*94*, *179*) site and form dystrophic end bulbs (*180*). The lack of adequate regeneration in the adult CNS following injury results in permanent functional deficits. Major factors hindering axon regeneration include a poor intrinsic capacity of severed axons to grow (*106*, *181*), a long-lasting inflammatory response (*182*) and the lesion extracellular environment that contains molecules which restrict neurite outgrowth and plasticity such as CSPGs (*179*, *183*) and myelin-associated proteins (*i.e.* Nogo , MAG and OMgp) (*184–186*), as well as scar formation.

Scar formation characterized by a glial component surrounding a fibrotic component, does not occur in neonatal rodents less that 8 days old when injured, as no deposition of collagen and only transient gliosis are observed. The change to mature scarring occurs from around postnatal days 8–12 in rodents when deposition of collagen and formation of a glial scar around a fibrotic lesion core containing fibroblasts and macrophages can be identified in CNS

wounds (*159*, *187*, *188*).

The fibrotic lesion core of the adult CNS scar contains growth-promoting substrates for re-growing axons, such as laminins, fibronectin and collagen IV (*100*, *138*, *149*, *189–193*). Nonetheless, the excessive deposition of these basement membrane proteins is thought to create a binding matrix for a plethora of axon growth inhibitory molecules (*32*), including chondroitin, keratan and heparan sulfate proteoglycans, tenascin-C, EphB2 and semaphorin 3A (*86*, *97*, *144*, *149–151*, *194–197*), that accumulate at the lesion site to high local concentrations. Therefore, the overall fibrotic scar milieu is thought to weakly support or inhibit regrowth of injured axons.

Pharmacological modification or removal of certain ECM components present at the lesion site has proven some success to improve axonal regeneration after SCI and brain lesions (*32*, *146*, *148*, *198*, *199*). For example, anti-scarring treatment combining the iron chelator BPY-DCA, which temporarily suppress collagen IV biosynthesis, and cAMP, which inhibits proliferation of meningeal fibroblasts, reduces collagen deposition at the lesion core and enhances axon regeneration after SCI and brain lesions (*148*, *199*). Additionally, treatment with the iron chelator deferoxamine and inhibition of lysyl oxidase, a key enzyme in collagen biosynthesis, leads to improved axon regeneration and accelerates recovery after SCI (*152*, *200*).

Stabilization of microtubules with antimitotic agents such as taxol or Epothilone B prevents dystrophic growth cone formation and is accompanied by reduced fibrotic scarring, enabling axonal regeneration and functional recovery (*139*, *201*).

Stromal fibroblasts in the lesion core of CNS lesions express the chemorepellent molecule semaphorin 3A that inhibits outgrowth of injured neurites (*144*, *150*, *151*, *202*). Inhibition of semaphorin 3A leads to robust axon regeneration after spinal lesions (*203*). Stromal fibroblasts also upregulate the expression of EphB2 receptor after SCI (*86*). Conditional deletion of the inhibitory axonal guidance molecule and corresponding ligand ephrin B2 in scar-forming astrocytes decreases gliosis, and improves axonal regeneration and motor recovery after SCI (*204*). Additionally, TGF-β1 drives fibrotic scarring by binding to type I and type II TGF-β receptors that are strongly induced in stromal fibroblasts after CNS injury (*156*). Application of TGF-β1 inhibitors or neutralizing antibodies was also shown to reduce the generation of fibrotic scar tissue after cerebral wounds and promote axon regeneration (*205–207*).

4 STROMAL FIBROBLASTS - PERICYTES AS A CANDIDATE CELL OF ORIGIN

Fibrotic tissue generation, where excessive ECM proteins are deposited by a large number of fibroblasts, is a major feature of scarring and fibrosis across all organs and in diverse types of pathology (*27*). Although recognized for a long time, the source of CNS scar-forming stromal fibroblasts has been difficult to establish (*79*).

Recent studies have implicated pericytes and perivascular fibroblasts as the major source of ECM-producing (myo)fibroblasts found in conditions such as kidney, lung and liver fibrosis, and dermal and muscle scarring (*208–213*).

4.1 CNS PERICYTES

Although Eberth described the existence of pericytes in 1871 (*214*), the discovery of a population of contractile cells surrounding the endothelial cells of blood vessels is usually attributed to the French scientist Charles-Marie Rouget (*215*). The term 'pericyte' was later coined by Zimmermann in 1923, referring to their proximity to endothelial cells. Based on morphology, location and function, he proposed the existence of multiple subsets of pericytes (*216–218*)

CNS pericytes are vascular mural cells surrounding blood microvessels of the CNS. They are embedded within the vascular basement membrane and send cytoplasmic processes along the abluminal surface of the endothelial tube of capillaries, pre-capillary arterioles and post-capillary venules (*219*). Another kind of mural cells, termed vascular SMC, enwraps large diameter vessels, such as arteries and veins. Collectively, pericytes and microvascular SMC are called microvascular mural cells, or MMCs. Other non-mural periendothelial, or perivascular, cells include fibroblasts, adventitial cells and macrophages. Under homeostatic conditions, pericytes are seemingly present along all blood microvessels, but normally asbsent in lymphatic capillaries. Coverage of the vasculature by pericytes is the highest in the CNS, with about 30% of the endothelial tube surface being covered by pericytes (*220*, *221*).

Pericytes establish a 'peg and socket' type contact with endothelial cells at discrete points of discontinuous vascular basement membrane. Adhesion plaques and occluding type contacts have also been described. Additionally, gap junction-like structures have been reported between pericytes and endothelial cells (*222*, *223*).

4.1.1 Multifunctional role

The NVU is comprised of a group of vascular cells (mural cells and endothelial cells), glial cells (astrocytes, oligodendrocytes and microglia) and neurons that work in consonance to regulate cerebral blood flow and maintain the integrity of the BBB/BSB (*33*, *224*, *225*). Due to their strategic location in the NVU, between endothelial cells, astrocytes and neurons, pericytes serve as a hub, receiving signals from their surrounding cells, and convey functional responses that are indispensable for proper CNS functioning (**Figure 4A**,**B**).

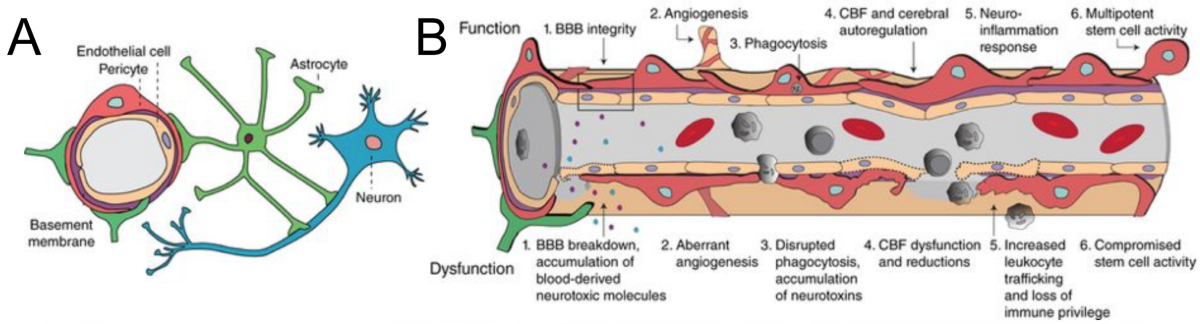

Figure 4 | The multifunctional role of CNS pericytes
(**A**) Simplified diagram of the NVU at the level of brain capillaries, highlighting the communication between endothelial cells, pericytes, astrocytes and neurons.
(**B**) Roles of pericytes under physiological conditions (top row) and consequences of pericyte dysfunction at the NVU (bottom row)
Reprinted by permission from Springer Nature (*219*), © 2016.

CNS pericytes play a vital role in the maturation, maintenance and permeability of BBB/BSB (*226–228*). This diffusion barrier is composed by an uninterrupted monolayer of endothelial cells that establishes continuous crosstalk with mural cells and astrocytes within the NVU (*219*, *229*). It limits paracellular and transcellular endothelial transport of various macromolecules and toxins from the blood to the brain. Pericytes control BBB permeability, in part by regulating bulk-flow transcytosis of fluid-filled vesicles across the BBB (*226–228*, *230*). Additionally, pericytes are implicated in the regulation of angiogenesis and stabilization of the microvasculature during CNS development and vascular remodeling (*33*, *222*, *231*). Whether pericytes are involved in the regulation of capillary blood flow is still under debate (*232–235*).

Among other functions, pericytes are involved in the clearance of amyloid-β peptide, as shown in a murine Alzheimer's disease model (*236*) and can display phagocytic activity *in vivo* (*228*, *236*, *237*). Under cytokine-mediated inflammation, neutrophils traffic through the venular walls at specific exit points, coinciding with areas of low pericyte coverage and discontinuous basement membrane (*238*, *239*).

A number of studies have associated pericyte degeneration with breakdown of the BBB/BSB and accumulation of blood-derived neurotoxic molecules into the CNS parenchyma (*228*, *230*, *240–242*). Interestingly, pericytes were recently shown to support neuronal survival through production of the neurotrophic growth factor pleiotrophin (*242*). Following cerebral

ischemia, pericyte *rigor mortis* leads to constriction of capillaries and obstruction of capillary flow (*235*, *243*, *244*). Moreover, pericytes were suggested as the source of MSCs from various human organs (*245*). While it is clear that not all pericytes behave as MSCs, whether all MSCs are pericytes awaits further investigation (*246*).

4.1.2 Heterogeneity

There is increasing evidence of a continuum of heterogeneous mural cell types along the microvascular tree. In particular, several pericyte subsets have been acknowledged based on morphology. Recent studies of the cortical angioarchitecture have identified thin-strand helical pericytes and mesh pericytes on capillaries and a multitude of transitional MMC phenotypes along the microvascular tree (**Figure 5**). Those include VSMC–pericyte hybrids, termed ensheathing pericytes, at the interface of pre-capillary arterioles and capillaries. Additionally, mesh pericytes with stellate morphology were identified at the interface of post-capillary venules and capillaries (*218*, *247–249*).

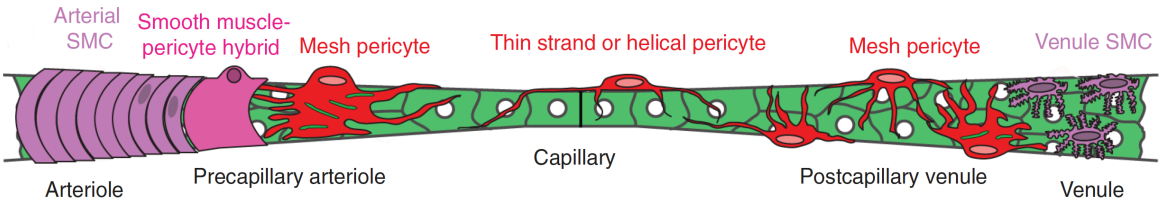

Figure 5 | Mural cell heterogeneity and organization in the adult CNS
Schematic depiction of the continuum of mural cell types along the cerebrovasculature in the mouse. Reproduced from (*247*).

A hindering factor regarding pericyte identity is the lack of a single entirely pericyte-specific molecular marker. Currently used markers are not exclusively expressed by pericytes, and do recognize additional cell types. An example is the commonly accepted pericyte marker NG2, which also marks OPCs, Schwann cells and macrophages after injury (*10*, *250*, *251*). In addition, no molecular marker recognizes all pericytes (*217*, *222*, *252*). Importantly, other cells than MMCs are present in perivascular spaces, including fibroblasts, adventitial cells and macrophages, and challenge their proper identification (*31*, *247*, *253*, *254*).

Heterogeneity among pericytes based on differential molecular marker expression has been previously recognized. The most well established molecular markers expressed by capillary pericytes are the cell surface antigens PDGFRβ, NG2, CD13 (also known as aminopeptidase-N) and RGS5 (*248*, *252*, *255*), also shared by other MMCs. Additional markers include the potassium inwardly-rectifying channel protein Kir6.1, a pore-forming subunit of ATP-sensitive potassium channels, and SUR2. New markers enriched in capillary pericytes have been suggested recently (*255*) and include vitronectin and IFITM1. It is still debatable whether pericytes express αSMA, a contractile protein that marks arterial SMCs (*222*, *252*,

255, *256*). Nonetheless, pericytes were recently shown to express moderate-to-robust levels of other contractile proteins, such as desmin, calponin-2 (*255*) and MYL9 (*257*).

Pericyte marker expression may be spatiotemporally regulated in conjunction with developmental stages, tissue types, pathological situations and *in vitro* or *in vivo* conditions, among others. Therefore, pericytes are ususally identified based on morphological characteristics and location (*e.g.*, encasement by the vascular basal lamina and extension of cytoplasmic processes along the abluminal surface of the endothelial tube), in combination with at least two molecular markers (*222*).
Functional heterogeneity among pericytes, vascular SMCs and perivascular cells in general, might also be explained by distinct developmental origin (*258*). Pericytes in the face, brain, and thymus have a neural crest origin (*259–266*), whereas pericytes of the lung, gut, liver and heart originate from the mesothelium (*222*, *267–272*).

Although heterogeneity among pericytes, and mural cells in general, can be appreciated, whether particular subsets of pericytes play different functions under physiological conditions or in response to an insult is still a matter of investigation.
The work presented in this thesis explores the contribution and role of a subset of pericytes in the context of CNS injury and disease.

5 PRESENT INVESTIGATION

5.1 AIMS

Paper I - To determine the cellular origin and function of fibrotic scar tissue following CNS injury

Paper II - To investigate the therapeutic potential of reducing fibrotic scarring by a subset of pericytes

Paper III - To investigate whether scarring by a subset of pericyte-derived cells is a conserved mechanism across diverse CNS lesions

Paper IV - To delineate the recruitment mechanism leading to fibrotic scar tissue generation by a subset of pericytes

5.2 PAPER I - RESULTS AND DISCUSSION

In the adult mammalian CNS, scar tissue that develops at sites of injury is composed of a fibrotic lesion core immediately surrounded by a glial scar. Before our work (**Paper I**), the cellular origin of stromal fibroblasts residing in fibrotic scar has not been addressed with genetic lineage tracing. In **Paper I** we have explored the contribution of pericytes and progeny in CNS scar formation after SCI by genetic fate mapping to overcome limitations related to injury-induced cell fate changes and shifts in marker expression.

To genetically label and fate map a subset of pericytes in the adult mouse spinal cord, we made use of BAC transgenic mice expressing a tamoxifen-inducible form of Cre-recombinase (*i.e.* CreER^{T2}) under the GLAST promoter (*273*), in combination with a Cre-reporter line expressing YFP (*274*), hereafter referred to as GLAST-CreER^{T2}; Rosa26-YFP mice (**Figure 6**).

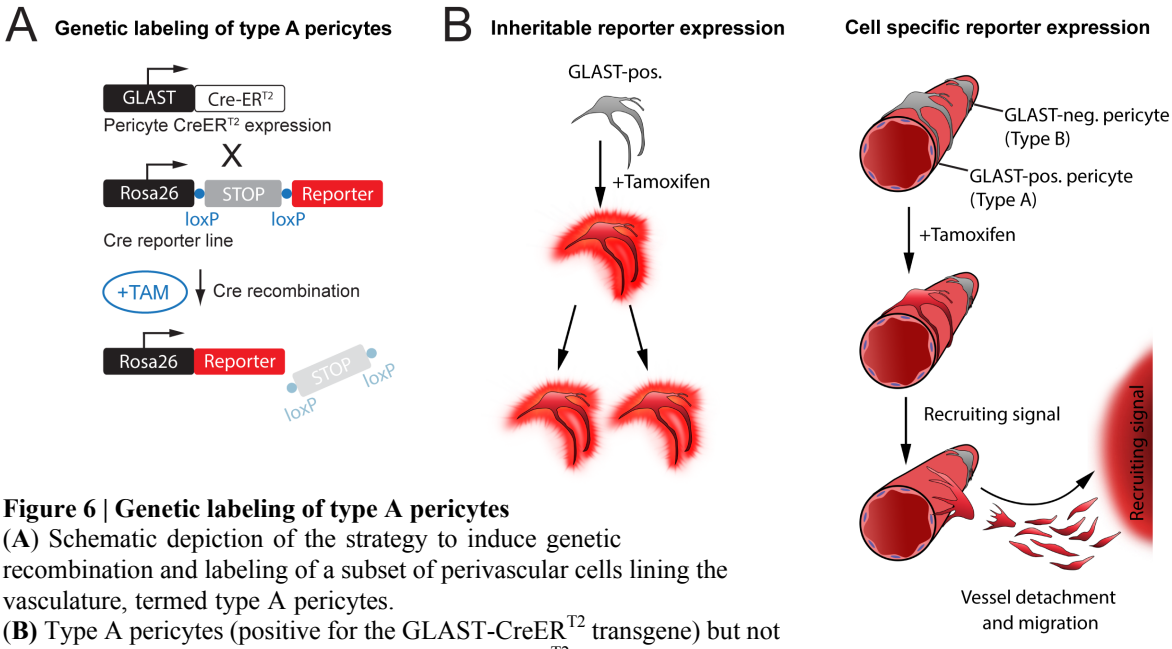

Figure 6 | Genetic labeling of type A pericytes
(**A**) Schematic depiction of the strategy to induce genetic recombination and labeling of a subset of perivascular cells lining the vasculature, termed type A pericytes.
(**B**) Type A pericytes (positive for the GLAST-CreER^{T2} transgene) but not type B pericytes (negative for the GLAST-CreER^{T2} transgene) undergo tamoxifen-mediated genetic recombination and turn on fluorescent Reporter expression. Type A pericytes and progeny can be traced by stable and inheritable labeling with fluorescent Reporter. Adapted from (**Paper IV**). Image credits: Jannis Kalkitsas

Upon tamoxifen-mediated genetic recombination, GLAST-expressing pericytes could be readily observed as YFP-positive cells lining blood vessels throughout the spinal cord parenchyma. Thorough characterization of the GLAST-CreER^{T2}; Rosa26-YFP line at the ultrastructural level and differential marker expression revealed specific targeting of a distinct pericyte subpopulation, which accounts for about 10% of all PDGFRβ-expressing pericytes within the uninjured adult mouse spinal cord parenchyma. Pericyte heterogeneity based on marker expression and morphology has been recognized but somehow difficult to ascertain (*275*). We have termed the recombined subclass of pericytes labeled in the GLAST-CreER^{T2}; Rosa26-YFP line, type A pericytes, and the other non-recombined subpopulation(s) of pericytes, as type B pericytes. Type A pericytes expressed the established pericyte markers

PDGFRβ and CD13, but not desmin and αSMA, found in type B pericytes. Additionally, some of the GLAST-positive pericytes also expressed PDGFRα. Apart from type A pericytes, occasional recombination was observed in association with the meningeal vasculature and in a minor subset of ependymal cells and white matter radial astrocytes in the spinal cord (*30*).

Genetic pericyte labeling in combination with SCI revealed that type A pericytes strongly reacted to injury by increasing in number. Recombined pericyte-derived cells proliferated massively and peaked at 9-14 days post injury and then decreased in number as the scar condensed and matured. During the wound contraction phase, type A pericyte-derived cells were found to transiently express the myofibroblast marker, αSMA. Interestingly, in response to injury, a large fraction of type A pericyte-derived cells dissociated and migrated away from the blood vessel wall, and gave rise to stromal fibroblasts that ultimately clustered in the core of the lesion and became embedded in fibrous ECM. Type A pericyte-derived cells remained in the lesion core for at least 7 months after SCI and were chronically surrounded by reactive astrocytes. Importantly, only type A pericytes, but not type B pericytes, leave the blood vessel wall, demonstrating functional heterogeneity among pericyte subpopulations regarding scar formation. As stated earlier, some type A pericytes expressed PDGFRα, a marker that is shared with OPCs. We have, therefore, verified whether type A pericytes gave rise to oligodendrocytes or OPCs after SCI. Fate mapping of type A pericytes, including PDGFRα-positive cells, extending several months after injury, showed no contribution to oligodendrocyte-lineage cells. On the other hand, co-targeting of scar-forming pericytes and OPCs was reported in recent lineage tracing studies employing PDGFRα-CreER^{T2} lines (*10*) and needs to be taken into consideration when interpreting results (*276*).

In summary, we showed that a discrete subpopulation of perivascular cells lining the vasculature, termed type A pericytes, are the primary source of stromal fibroblasts that form the core of the chronic CNS scar in the injured mouse spinal cord (**Figure 7**). These observations are in line with a pericyte/perivascular fibroblast origin of fibrotic tissue in peripheral organ fibrosis (*208–213*, *277*, *278*).

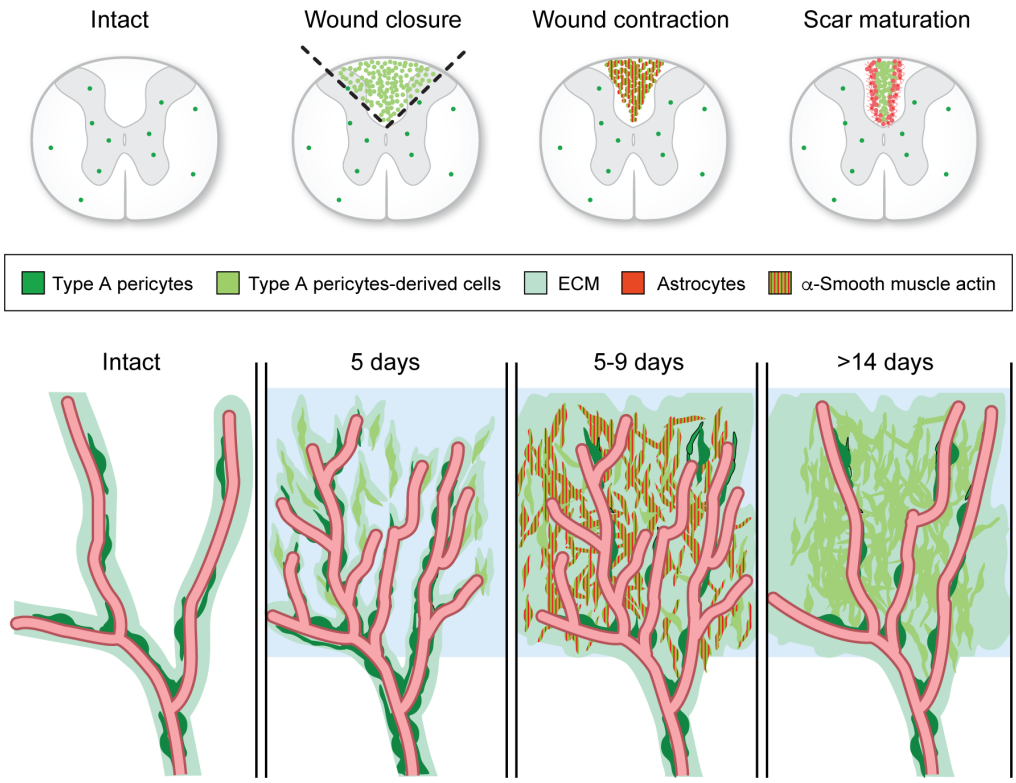

Figure 7 | Type A pericyte-derived scarring after SCI
In the uninjured CNS, type A pericytes represent a small subpopulation of perivascular cells associated with the vasculature within the grey and white matter. After injury, type A pericytes invade the lesion site together with angiogenic vessels, proliferate, dissociate from the vascular wall and gather at the lesion site embedded in fibrous ECM. During the wound contraction phase, type A pericyte progeny temporarily turn on αSMA expression. By the end of the wound closure process, type A pericyte-derived scar tissue occupies the lesion core and is bordered by reactive astrocytes. As the scar matures, a sharp border is established and segregates the glial and fibrotic compartments of the scar. With time, the scar further condenses and reduces in size but remains chronically.
Reproduced from (*79*).

Following injury, we observed that nearly all scar-participating pericyte-derived cells have been generated through proliferation. To investigate the role of pericyte-derived scarring following SCI, we made use of a genetic tool that allowed cell-specific and inducible inhibition of the generation of type A pericyte progeny by specifically blocking injury-induced proliferation. This was achieved by creating a new transgenic mouse line that result from crossing GLAST-CreER^{T2}; Rosa26-YFP mice with 'Rasless' conditional knockout mice, hereafter referred to as GLAST-CreER^{T2}; Rasless; Rosa26-YFP mice (**Figure 8**). The 'Rasless' line (*279*) is a complete knockout for the H-*ras* and N-*ras* genes and a conditional knockout for the K-*ras* gene, important players in mitogenic signaling and proliferation. Tamoxifen-induced recombination in GLAST-CreER^{T2}; rasless; Rosa26-YFP mice resulted in the loss of H-*ras*, N-*ras* and K-*ras* genes specifically in type A pericytes and conferred less proliferative capacity to this population of cells upon injury. As control, we used animals with identical genotype that were treated with vehicle and, therefore, did not undergo Cre-mediated recombination.

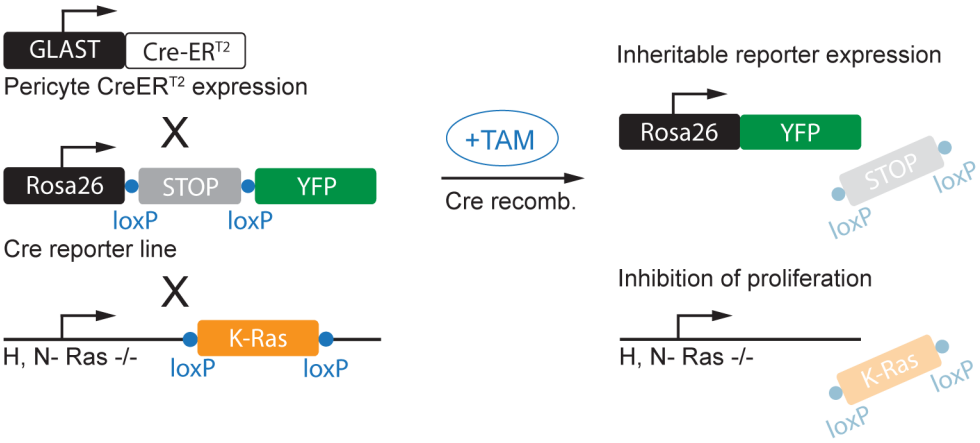

Figure 8 | Genetic strategy to block the generation of progeny by type A pericytes
Adapted from (*280*).

Following SCI, control animals preserved intact type A pericyte-derived scarring and presented prominently packed scars with a dense lesion core composed of PDGFRβ-expressing fibroblast-like cells enclosed in fibronectin, and lined by reactive astrocytes. Conversely, in animals presenting nearly complete inhibition of type A pericyte proliferation (strong inhibition, **Figure 9**), fibrotic scarring was abolished and the lesion site failed to seal. These animals developed an open tissue defect at the site of the injury. These observations demonstrated that type A pericyte-derived cells are essential to patch off the injured tissue and are required for the reestablishment of tissue integrity. Interestingly, in cases of incomplete Cre-mediated inhibition of type A pericyte proliferation (moderate inhibition, **Figure 9**), fibrotic scarring was reduced, as seen by a less dense scar core presenting significantly fewer PDGFRβ-expressing stromal cells and reduced fibronectin deposition, but allowed simultaneous sealing of the wound. Moderate inhibition of type A pericyte proliferation thus represents an attractive scenario to assess the influence of pericyte-derived fibrotic scarring on axonal regeneration and functional recovery (**Paper II**).

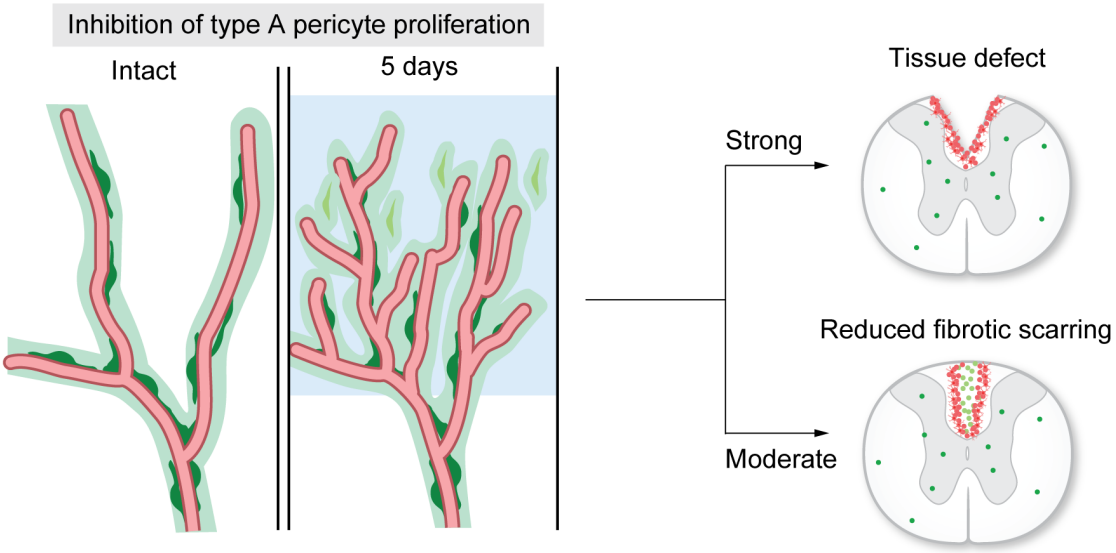

Figure 9 | Inhibition of type A pericyte proliferation results in reduced fibrotic scar tissue generation
Strong reduction of type A pericyte proliferation abolishes fibrotic scar tissue generation and results in an unsealed lesion. Conversely, moderate inhibition of type A pericyte proliferation allows wound closure and leads to reduced fibrotic scar tissue generation.
Reproduced from (*79*).

5.3 PAPER II - RESULTS AND DISCUSSION

The identification of type A pericytes as the main source of scar-forming stromal fibroblatsts contributing to fibrotic tissue generation in the injured spinal cord (**Paper I**) enabled us to selectively address how pericyte-derived fibrotic scarring influences axon regeneration and functional recovery following SCI.

In **Paper II**, we employed the same inducible and cell type specific genetic strategy to render scar-forming type A pericytes unable to proliferate (*i.e.* GLAST-CreER^{T2}; rasless; Rosa26-YFP mice). We showed, by RNA-sequencing, that the lesion enviroment following a dorsal hemisection SCI was less fibrotic when pericyte-derived scarring was attenuated (moderate inhibition, **Figure 9**) compared to conditions of intact pericyte-derived scarring, in which excessive deposition of fibrous ECM could be appreciated. Animals with nearly complete ablation of pericyte proliferation, leading to impaired sealing of the lesioned tissue (strong inhibition, **Figure 9**), were excluded from the analysis. Moderate reduction of type A pericyte-derived fibrosis led to decreased axonal dieback and significantly increasead regeneration of descending CST axons into and beyond the lesion site, after SCI. As expected, control animals with intact pericyte-derived scarring showed densely packed fibrotic scars with minimal to no regeneration of CST axons beyond the lesion site. Similarly, increased density of RST axons innervating the spinal cord ventral horn caudal to injury was detected in animals with reduced pericyte-derived scarring when compared to control animals. Using optogenetic stimulation of the CST (*281–284*) combined with electrophysiology, we validated that CST regenerated fibers synaptically integrated into the

local spinal circuitry below the lesion site. In addition, improved recovery of sensorimotor function in a horizontal ladder-walking test (*285*), catwalk-aided gait analysis and optogenetic motor-task was observed when pericyte-derived scarring was attenuated (**Figure 10**).

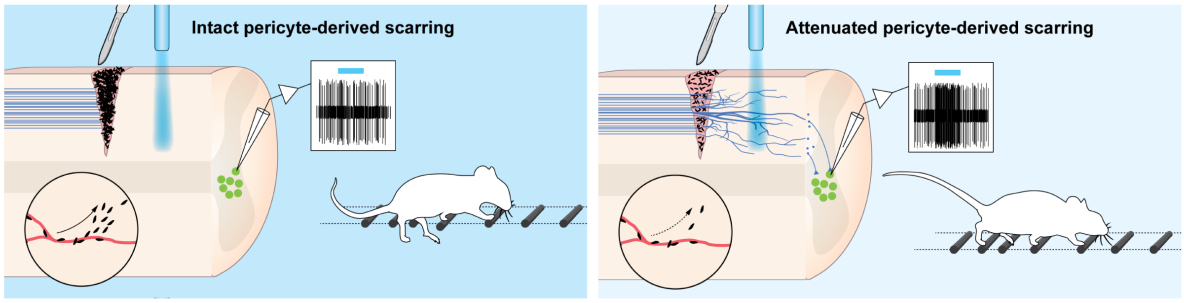

Figure 10 | Genetic labeling of type A pericytes
Attenuation of type A pericyte-derived scarring promotes axon regeneration and functional recovery after SCI. Regenerated fibers make functional synapses with local spinal neurons below the lesion site.
Reproduced from (*280*).

In general, sensory axons possess greater regenerative ability than retinal ganglion cell and central motor axons (*286*, *287*). The fibrotic matrix that develops at the injury site can be penetrated to a certain extent by some classes of re-growing ascending sensory axons, but not by re-growing descending motor axons (*288*). It remains to be tested whether attenuation of pericyte-derived scarring favors injured sensory axons to cross lesions scars and regenerate distally. In contrast to CST axons, which are myelinated (*289–291*), serotonergic RST axons are unmyelinated (*292*, *293*) in rodents. Additionally, serotonergic axons exhibit minimal axonal dieback and enhanced ability to regenerate or sprout after CNS injury, when compared to CST axons, which are very refractory to regeneration (*193*, *294–296*). We decided to study regeneration of serotonergic RST axons because of their relevance in modulating the activity of spinal motor systems (*189*), and regeneration of the CST due to its significance in clinical neurorehabilitation. We observed that attenuation of pericyte-derived scarring reduced fibrosis and modified the lesion environment, facilitating RST and CST axon regeneration, and promoted sensorimotor recovery after SCI. Interestingly, improved performance in sensorimotor behavioral tasks was positively correlated to RST and CST axon regeneration, suggesting that regenerated fibers may play a role in behavioral recovery, probably through indirect relay-circuits (*297–300*). Our observations that fibrotic scar tissue inhibited axonal regeneration are in agreement with other reports. In addition to enhancing the capacity of axons to grow, microtubule-stabilizing drugs were shown to decrease fibrotic scarring and promote regeneration of RST and sensory axons after SCI (*139*, *201*, *301*, *302*). Additionally, when stimulating the intrinsic growth capacity of neurons by PTEN deletion, axon regeneration occurs along glial bridges and in regions devoid of fibrotic tissue (*106*, *107*). Further studies will be required to investigate whether upregulation of genes associated with axon outgrowth, cell protection, and neural development occurs in cortical pyramidal neurons or hindbrain raphespinal neurons upon reduction of pericyte-

derived scarring, as suggested by others after anti-scarring treatment (*303*, *304*) or CSPG modulation with chondroitinase ABC (*305*, *306*).

We showed that, in conditions of intact pericyte-derived scarring, only a small percentage of CST fibers reached the lesion core, making contact with pericyte-derived cells, at sub-acute stages after SCI. Since a glial scar rapidly flanked the injured tissue, most of the CST fibers contacted GFAP- and NG2-expressing glia and, to a less extent, CD68-positive microglia/macrophages. It is intuitive to think that pericyte-derived fibrotic tissue would only represent a molecular and physical barrier to regeneration of severed axons that are able to overcome macrophage-mediated axonal dieback (*307*), penetrate the glial-fibrotic scar interface, and finally reach the lesion core. However, since pericyte-derived cells intermingle with macrophages in the lesion core of the scar and participate in the establishment of a sharp border with glial cells, alterations in the fibrotic compartment of the scar will likely influence the behavior of other scar-participating cells. It is well appreciated that prevention, deletion or attenuation of ependymal cell and astrocyte scarring results in impaired functional recovery that is coupled to increased neurotoxic inflammation, augmented fibrotic scarring and enlargement of the lesion core, and decreased axon regeneration (*35*, *41*, *43*, *100*, *308*). We observed reduced astrogliosis and inflammation, but no significant changes in the number of OPCs, when pericyte-derived scarring was attenuated after SCI. These changes may have contributed positively to the improved axonal regeneration and functional recovery observed in our study. Reports by others have backed up this notion. For example, animals with reduced glial and fibrous scarring and a dampened inflammatory response show improved axon regeneration coupled to, in some cases, improved recovery after SCI (*76*, *81*, *88*, *207*, *309–311*). Similarly, disrupting reactive astrocyte-collagen I interactions, likely produced by pericyte-derived fibrotic cells, attenuates glial scarring and leads to improved axon regrowth and functional recovery after SCI (*87*). Conversely, excessive fibrotic scar formation, with pronounced deposition of fibronectin, laminin and collagen IV, and increased expression of CSPGs at the lesion site are correlated to worsened locomotor performance after SCI (*312*).

Scar modulation treatment by inhibition of collagen IV biosynthesis decreases basement membrane deposition and promotes axon regeneration after brain injury when applied acutely and early sub-acutely after injury (*313*). However, when administered at late sub-acute or chronic stages after injury, it fails to decrease matrix deposition and does not promote regeneration of axons across the injury site. These observations show that interfering with collagen IV biosynthesis needs to be completed at early stages after injury, when fibrotic scar tissue is being generated. It suggests that, at later stages, pericyte-derived scar tissue has been already formed and, is, therefore, insensitive to inhibition of collagen IV biosynthesis.

A recent study showed that ablation of dividing NG2-expressing cells after SCI led to loss of integrity of the glial scar, prevented injury-induced angiogenesis and abolished fibrotic scar formation, with concomitant increase in axon regrowth. These changes resulted in persistent edema and hemorrhage, and animals displayed worsened functional recovery

after SCI (*75*). The NG2 promotor targets both OPCs and pericytes and it is not possible to distinguish whether the impaired functional recovery and morphological changes in scar tissue observed after ablation of proliferating NG2-expressing cells are due to selective killing of NG2-expressing pericytes or NG2-expressing glia, or both. In another study, specific ablation of capillary pericytes co-expressing NG2 and PDGFRβ with diphtheria toxin caused BBB breakdown, accompanied by disturbances in blood flow and neuronal loss (*242*). In our study (*280*), we selectively targeted type A pericytes and not a mixed population of cells types. Additionally, we did not ablate proliferating type A pericytes. Instead, employing GLAST-Rasless-YFP mice, we mediated cell-specific and inducible inhibition of type A pericyte proliferation, and, therefore, noticed a reduction in the generation of injury-responsive type A pericyte-derived progeny. Importantly, the vasculature outside the lesion area remained unaffected (*30*).

As recognized for astrocytes (*35*, *43*, *100*) and ependymal cells (*41*, *308*), pericyte-derived scarring is necessary to preserve tissue integrity at acute and sub-acute stages after trauma, but represents an absolute barrier for axon regrowth at more chronic stages after SCI. We conclude that moderate reduction of type A pericyte-derived scarring promotes axon regeneration and functional recovery after SCI.

5.4 PAPER III - RESULTS AND DISCUSSION

Traumatic injuries to the brain and spinal cord, as well as, inflammatory demyelinating disease result in the formation of a permanent scar that exhibits a fibrotic and glial component. Scarring, accompanied by extensive vascular remodeling, can also be appreciated in brain tumors and after transient interruption of blood flow to the brain, followed by reperfusion (**Figure 3**) (*123*). Multiple cell types, including meningeal and perivascular fibroblasts, mesenchymal perivascular cells, fibrocytes and pericytes, have been suggested to contribute to ECM-producing stromal fibroblasts residing in fibrotic CNS scar tissue (*3*, *30*, *151*, *153–156*, *158–160*, *172*, *199*, *31*, *202*, *81*, *88*, *121*, *129*, *131*, *143*, *147*). However, no genetic fate mapping has been employed in those studies, making it difficult to ascertain the cellular origin of fibrotic scarring.

In **Paper III** we investigated the contribution of type A pericytes to scar-forming fibroblasts following penetrating and non-penetrating SCI, stab lesions to the brain, EAE, ischemic stroke and brain cancer. As previously, we employed GLAST-CreER^{T2};R26R-YFP mice to lineage-trace type A pericytes in the adult mouse CNS (**Figure 6**). In addition to the spinal cord, type A pericytes, sharing similar marker expression and comprising about 10% of all PDGFRβ-expressing cells, could be identified in the uninjured brain (**Figure 11A**).

We showed that type A pericytes are the main source of scar-forming fibroblasts that reside in fibrotic scar tissue generated following penetrating and non-penetrating traumatic SCI, cortico-striatal stab lesions and EAE. In response to injury, pericyte-derived cells migrated

away from the vascular wall and formed the fibrotic core of the lesion, bordered by reactive astrocytes (**Figure 11B**).

Tumor vessels are known to be leaky and tortuous, and present low pericyte coverage. As expected, type A pericyte coverage was decreased in tumor-associated vessels, in a GBM tumor model. Nonetheless, type A pericyte-derived cells dissociated from the vascular wall and contributed to tumor-associated stromal fibroblasts (**Figure 11C**).

The ischemic lesion core that developed following transient MCAO-induced striatal ischemia (striatal stroke) underwent profound vascular remodeling but lacked typical stromal fibroblasts, readily found in fibrotic scar tissue generated after SCI, TBI and EAE. Therefore, type A pericytes expand and contribute to increased vessel coverage, but were rarely detected away from the vascular wall after striatal strokes (**Figure 11D**). Interestingly, PDGFRβ-expressing stromal fibroblasts cells are promptly observed following cortico-striatal ischemic strokes (*121*). These observations suggest that the location and magnitude of the ischemic insult dictates the generation of scar-forming fibroblasts. It remains to be evaluated whether type A pericytes contribute to stromal fibroblasts following cortico-striatal ischemic strokes.

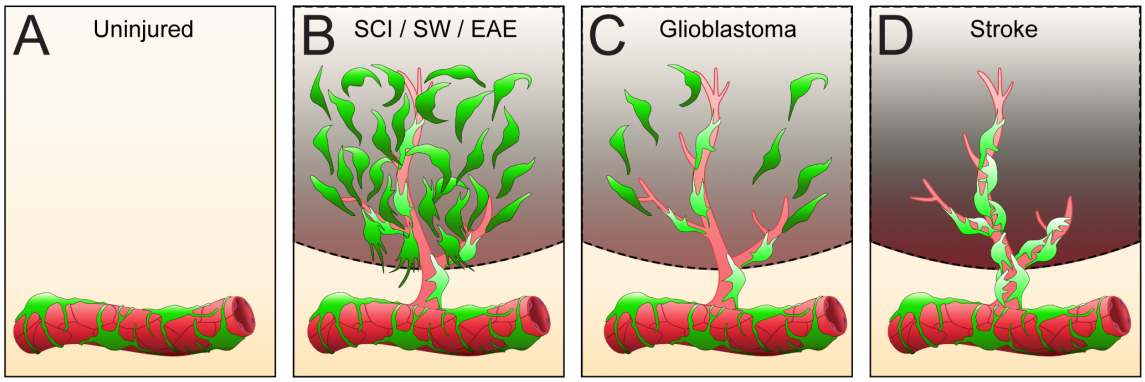

Figure 11 | Pericyte-derived fibrotic scar tissue formation is a conserved mechanism in response to diverse CNS lesions

Schematic illustration depicting the contribution of type A pericyte-derived cells to diverse CNS lesions. In the uninjured CNS, type A pericytes are found associated with the blood vessel wall (A). After penetrating and non-penetrating SCI, cortico-striatal stab lesions and EAE, type A pericytes give rise to progeny that dissociate from the vascular wall and cluster at the core of the lesion, making up the fibrotic scar (B). The vasculature of glioblastoma tumors shows reduced coverage by type A pericytes. Type A pericytes and progeny contribute to stromal fibroblasts in glioblastoma tumors (C). Following ischemic stroke confined to the striatum, type A pericytes increase in number but remain associated with the vascular wall (D). .

From (**Paper III**). Image credits: Jannis Kalkitsas

As appreciated in **Papers II** and **IV** the response of type A pericyte to CNS injury is closely linked to inflammation. Attenuation of type A pericyte-derived scarring is accompanied by reduced inflammation after SCI (**Paper II**). The contrary is also true, and preventing CCR2-dependent infiltration of monocyte-derived macrophages into the injured CNS results in reduced fibrotic scar tissue generation following SCI (**Paper IV**; (*76*)). We postulated that similar crosstalk between macrophages and pericytes takes place after TBI, EAE and GBM. However, following an ischemic striatal stroke, there is evidence suggesting that microglia activation predominates over macrophage infiltration and that infiltrating peripherally-derived macrophages acquire a microglial-like phenotype once in the infarcted tissue (*314*).

Additionally, neutrophils are mainly recruited from nearby skull bone marrow and not from throughout the whole body, as commonly accepted, following ischemic stroke (*315*, *316*). Therefore, differences in the activation and recruitment of inflammatory and immune cells may also underlie the lack of type A pericyte dissociation from the vascular wall after stroke.

Our results showed that only large lesions to the brain, as cortico-striatal stab wounds, elicited fibrotic tissue generation and type A pericyte-derived scarring. Stabs lesions restricted to the cerebral cortex were accompanied by gliosis but were poor at producing fibrotic tissue, as indicated by the emergence of low or no fibroblast-like cells after injury. Therefore, type A pericyte-derived scarring was limited under these conditions. This is in agreement with a report revealing no increase in Col1a1-expressing fibroblasts following a cortical stab wound and, therefore, no participation of Tbx18-expressing mural cells (pericytes and vascular smooth muscle cells) in the injury response (*317*). Reevaluation of the contribution of Tbx18-expressing mural cells in the context of larger brain lesions, such as cortico-striatal stab lesions, should clarify the role of pericytes in fibrotic scar tissue formation. Recent data suggests that all vessel-associated perivascular cells express Tbx18 (*255*, *318*). Nonetheless, type A pericytes and Tbx18-expressing mural cells could still represent two functionally different perivascular populations, with scar formation properties being restricted to type A pericytes.

Fibroblasts migrating from the damaged *dura mater* meningeal layer (*144*, *150*, *151*) have for long been considered the primary source of fibrotic scar tissue in penetrating models of SCI. Since, in addition to type A pericytes, occasional recombination occurs in cells associated with the meningeal vasculature in the uninjured brain and spinal cord of GLAST-CreER^{T2};R26R-YFP animals (*30*), there is a chance that *dura mater* fibroblasts contribute to the fibrotic scar, following penetrating SCI. However, even following non-penetrating spinal injuries, which do not breach the *dura mater* and limit the invasion of *dura mater*-derived fibroblasts into the lesion site (*e.g.* contusion, clip compression and crush injuries (*28*, *106*, *107*, *136*, *301*, *319*, *320*)), extensive fibrotic tissue generation can be appreciated (**Figure 3A**,**E**), suggesting an alternative origin of scar-forming fibroblasts.
We compared the contribution of type A pericyte-derived scarring to penetrating and non-penetrating spinal lesions, by employing a DFI (*30*, *38*, *42*) and a complete crush SCI model (*106*, *107*), respectively. In case the fibrotic scar tissue is derived from recombined *dural* meninges, we would observe scar-forming fibroblasts recombined following DFI, but little or no scar-forming fibroblasts recombined after crush SCI. Conversely, if the majority of scar-participating fibroblasts originate from type A pericytes, we would observe similar numbers of recombined fibroblasts after DFI and crush SCI. Indeed, we found that the majority of stromal fibroblasts that compose the fibrotic lesion core were recombined following both DFI and spinal crush, and therefore derived from type A pericytes. These results suggested that type A pericytes, rather than *dura mater* meningeal fibroblasts, are the major source of scar-forming fibroblasts following penetrating and non-penetrating injuries to the spinal cord, shifting the origin of fibrotic scarring from meninges to the vasculature. However, we could not rule out that both meningeal fibroblasts and type A pericytes

contribute to stromal fibroblasts in penetrating spinal injuries. Future studies, employing a lentivirus-based technique to label meninges and meningeal substructures (*321*), could elucidate the role of meningeal-derived scarring after SCI.
Col1a1-expressing perivascular fibroblasts were proposed to function as the primary source of scar-forming fibroblasts following contusive SCI in mice (*31*). Since no fate mapping of Col1a1 cells has been employed in this study, the origin of scar-participating fibroblasts could not be pinpointed. Additional experiments are required to investigate whether type A pericyte-derived fibroblasts and Col1a1-expressing fibroblasts represent the same population. Following SCI, reactive astrocytes interacted with type A pericyte-derived fibrotic cells to form a sharp lesion border, separating the glial and fibrotic compartments of the scar. Similar segregation of fibrotic and glial components occurred when GFAP-positive glial processes bridged the lesion site, in regions devoid of pericyte-derived cells. Interestingly, type A pericyte-derived cells apposed to reactive glia at the glial-fibrotic lesion border exhibited a different morphology and arranged differently than type A pericyte-derived cells that intermingled with immune cells in the inner core of the fibrotic scar. It remains to be explored whether these two morphologically distinct populations of type A pericyte-derived cells are functionally different.

Type A pericytes are the major source of scar-forming fibroblasts following chronic EAE. Further studies will be required to investigate whether pericyte-derived scarring resolves once a lesion gets remyelinated. This could be tested in a relapse/remitting EAE model (*322*, *323*). Additionally, chemically-induced demyelination models, such as LPC-induced focal demyelination (*324*) and cuprizone-mediated systemic demyelination (*325*, *326*), with established dynamics of de- and re-myelination (*324*, *327*, *328*), could prove useful in answering this question.

Rats and humans, but not mice, are prone to develop fluid-filled cysts (cavitation) after contusive spinal injuries. Nonetheless, fibrous connective scar tissue enriched in ECM deposits and inflammatory cells forms at the lesion site after traumatic SCI in humans, specially after crush, compression and lacerating injuries (*3*, *62*, *137*, *139*, *140*). Interestingly, fibrotic scarring still occurs in contusion injuries containing multiple hemorrhagic and necrotic regions in humans (**Paper III**, (*3*, *62*, *137*). Corroborating our observations in the mouse, we found regions of non-neural scar tissue enriched in PDGFRβ-expressing stromal fibroblasts and delimited by reactive glia following traumatic SCI in humans. Additionally, perivascular aggregates of PDGFRβ–expressing stromal cells surrounded by reactive glia were observed in spinal cords of individuals with active MS, similar to what has been reported in MS brain lesions (*129*). In agreement with our observations following MCAO-induced ischemia in the mouse, PDGFRβ-expressing cells associated with the vasculature and exhibited characteristics of pericytes in the ischemic lesion core of stroke patients. Likewise, PDGFRβ-expressing pericytes remained in close association with blood vessels in the stroma of aggressive grade IV human GBM tumors.
Pericytes and perivascular fibroblasts have been identified as the main source of (myo)fibroblasts in peripheral organ fibrosis (*208–213*, *277*, *278*), attributing functional

similarities to pericytes in the CNS and peripheral organs.
In summary, we revealed that pericyte-derived fibrotic scarring is conserved in response to different CNS lesions, with the exception of striatal stroke. Humans also generate fibrotic scar tissue enriched in stromal fibroblasts after traumatic SCI and MS.

5.5 PAPER IV - RESULTS AND DISCUSSION

Differences in the molecular and cellular composition of distinct CNS regions may impact on the injury response (*329*). While studying the contribution of type A pericytes to a vast number of CNS insults we noticed that lesions leading to greater white matter damage had the tendency to generate larger amounts of fibrotic scar tissue. Namely, cortical stab wounds were poor at triggering fibrotic scar tissue formation (*317*), but deeper cortico-striatal stab lesions, extending through the *corpus callosum* white matter tract, potentiated pericyte recruitment and generation of fibrotic scar tissue. Similarly, larger ischemic insults, affecting both cortical and subcortical areas and damaging the *corpus callosum* white matter (*121*), or subcortical white matter infarction (*330*), showed extensive fibrotic scar tissue generation with large numbers of stromal fibroblasts and perivascular cells away from the vascular wall, in comparison to ischemic strokes restricted to the striatum (**Paper III**). Similarly, white matter injury was found to influence the behavior and reactivity of grey matter cellular components (*331*). These observations suggested that the extent of white matter damage may impact and titer pericyte-derived fibrotic responses. Therefore, in **Paper IV**, we explored the role of white matter damage and myelin in type A pericyte recruitment following CNS injury. We employed GLAST-CreER^{T2} mice (*30*, *273*, *280*) crossed to a Rosa26-tdTomato Cre-reporter line (*332*), hereafter referred to as GLAST-CreER^{T2};Rosa26-tdTomato, to fate map type A pericytes and progeny and started by investigating regional differences in pericyte-derived fibrotic scarring following discrete lesions to the spinal cord grey or white matter. Corroborating our initial hypothesis, these experiments showed that white matter lesions trigger a greater and more widespread recruitment of type A pericyte progeny when compared to grey matter lesions. Similar differences in white versus grey matter responses have been reported for other cells types. Following SCI, the density of microglia/infiltrating monocyte-derived macrophages increases by 40-fold in lesioned white matter against 9-fold in lesioned grey matter (*333*). Following discrete mechanical injuries, a larger inflammatory response in white matter compared to grey matter is observed within both the brain and spinal cord (*334*). Similarly, microglia proliferation is increased in the white matter compared to grey matter following cerebral stab lesions affecting the grey and white matter (*331*).

In addition to other molecular and cellular differences, white matter regions are enriched in their myelin and mature oligodendrocyte content when compared to grey matter regions. We subsequently injected LPC, a membrane-solubilizing agent that preferentially targets myelin-producing cells and causes minimal axonal damage (*324*), into the spinal cord white matter and found that focal myelin damage *per se*, uncoupled to a traumatic injury or

invasive mechanical tissue damage (*e.g.* spinal lesion), is sufficient to induce pericyte-derived scarring.

We then tested whether myelin itself, independent of chemically-mediated degeneration, was capable of initiating a fibrotic response. We found that injection of purified myelin into the uninjured spinal cord grey matter, but not cell membranes in general (*e.g.* isolated liver membranes), was able to recruit pericyte progeny from the vascular wall. When studying this process in more detail we observed that intraspinal myelin injection recapitulated most temporal and spatial aspects of CNS scar formation, including activation of microglia, infiltration of peripherally-derived macrophages, clearance of myelin debris by phagocytic cells, pericyte-derived cell recruitment from the blood vessel wall and fibrotic scar formation, followed by astrocyte scarring at later stages. Pericyte-derived cells intermingling with Mac2-expressing cells formed the fibrotic core of the lesion and were immediately surrounded by reactive astrocytes, as seen after SCI (*30*, *280*). In contrast, intraspinal injection of liver membranes triggered activation of microglia and gliosis but elicited nearly no recruitment of pericyte progeny and only minor infiltration of peripheral macrophages. Although it is clear that monocyte-derived macrophages are vital for clearance of myelin debris following CNS injury (*335*, *336*), the same process does not take place for clearing off liver membranes. Our results showed that cellular uptake and clearance of liver membranes was initially slow, but activated microglia were capable on their own to phagocytose non-CNS membrane components, suggesting that microglia failed to recruit peripherally-derived macrophages to assist in adequate and time efficient debris clearance (*337*). In agreement with previous observations recognizing activated neuroinflammatory microglia as potent inducers of astrocyte reactivity and polarization (*90*, *338*), we detected an astrogliotic response following intraspinal injection of liver membranes.

Interestingly, we noticed that a sharp increase in the number of Mac2-expressing cells, confirmed in subsequent experiments to correspond to the entry of peripherally-derived macrophages into the CNS, was accompanied by activation and recruitment of pericyte progeny from the vascular wall, starting at 3 days and peaking at 5 days post intraspinal myelin injection. Additionally, we found that the area covered by Mac2-expressing cells following CNS insults (*e.g.* discrete grey and white matter spinal lesions and intraspinal LPC or myelin injections) positively correlated to the extent of pericyte-derived scarring. We therefore examined whether recruitment of pericyte progeny was dependent on the infiltration of monocyte-derived macrophages. In mice lacking both alleles of CCR2 ($CCR2^{-/-}$), a receptor that is required for the infiltration of monocyte-derived macrophages into the inflammed CNS (*336*), recruitment of pericyte-derived cells was either abolished or significantly reduced following instraspinal injection of myelin or complete crush spinal cord injury, respectively, when compared to mice heterozygous for the CCR2 null alleles ($CCR2^{+/-}$). A recent study proposed that endothelial cells are capable of engulfing and degrading myelin, and regulate the recruitment and activation of macrophages, angiogenesis and fibrosis after SCI and EAE (*339*, *340*). Additional work is needed to reveal how this process regulates type A pericyte recruitment in CNS injury.

After confirming that myelin acts as a potent trigger of fibrotic pericyte responses, a process that is dependent on and titrated by the extravasation of monocyte-derived macrophages, we set out to dissect which myelin-associated proteins are involved in the recruitment of monocytes and pericyte-derived cells after CNS insult. We showed that structural myelin proteins such as PLP and MBP did not induce recruitment of pericyte progeny nor Mac2-positive cells following intraspinal injection. Next we tested myelin membrane proteins with extracellular domains capable of interacting with immune cells. We started by investigating the effect of Nogo because microglia and macrophages were shown to express Nogo receptors (*341*, *342*) and reduced recruitment of neutrophils and monocytes to sites of inflammation are detected in mice lacking Nogo-A/B (*343*). Nogo contains two extracellular domains, Nogo-66 and Nogo-A-Δ20, implicated in axon growth inhibitory effects via distinct receptors (*185*). Intaspinal injection of a Nogo-A-Δ20 peptide sequence (aa544-725), specific to Nogo-A (*344*), into the spinal cord grey matter did not trigger robust recruitment of Mac2-positive cells nor fibrotic pericyte progeny. Conversely, injection of a Nogo-66 peptide sequence (aa1026-1091), common to all Nogo isoforms (*344*), mediated extensive recruitment of Mac2-expressing cells and pericyte-derived cells. Additionally, Nogo has been implicated as a regulator of vascular remodelling (*345*, *346*), Nogo-A has been shown to inhibit developmental CNS angiogenesis (*347*) and blockage of Nogo-A pathway promotes vascular growth and maturation, and functional recovery after stroke (*348*). We therefore injected a peptide sequence corresponding to a region of the NogoA/B protein linked to vascular remodelling (aa 1-172) (*349*) into the uninjured spinal cord grey matter and found minor recruitment of pericyte-derived cells and Mac2-expressing cells.

We then explored the ability of MAG and OMgp, myelin-associated proteins involved in axon growth inhibition that also bind the Nogo-66 receptor (NgR1) (*185*), to trigger recruitment of pericyte-derived scarring and found widespread recruitment of Mac2-expressing cells and scar-forming pericytes. These experiments demonstrated that individual exposure of uninjured spinal cord to Nogo-66, MAG and OMgp is sufficient to induce fibrotic scarring. To investigate if endogenous myelin-associated proteins play a role in fibrotic scar tissue generation, we performed a complete crush SCI in mice lacking all known Nogo isoforms, MAG and OMgp (Nogo-, MAG-, and OMgp- null mutant mice) (*350*). We found that the lesion core volume and fibrotic scar tissue generation (Mac2-positive and PDGFRβ-expressing fibrotic cells) were reduced in Nogo-, MAG- and OMgp-single knockout mice and triple knockout mice ($Mag^{-/-}$;$Nogo^{-/-}$;$Omgp^{-/-}$) in comparison to control triple heterozygous mice ($Mag^{+/-}$;$Nogo^{+/-}$;$Omgp^{+/-}$), 7 days after SCI. In line with these results, NogoA/B deletion was found to reduce liver fibrosis (*351*, *352*).

In summary, we highlight the role of three well known axon growth inhibitory proteins associated with myelin in recruiting peripherally-derived macrophages and type A pericyte-derived progeny that contribute to fibrotic scar tissue formation after CNS injury (**Figure 12**). We hypothesized that, in addition to their known effects in alleviating axon growth inhibition, therapeutic agents interfering with Nogo-A signaling (*353–361*) or blocking the interaction of NgR1 with its ligands (*362–366*), may reduce fibrotic scarring, and contribute to the neuroregenerative effects and improved functional recovery perceived after therapy.

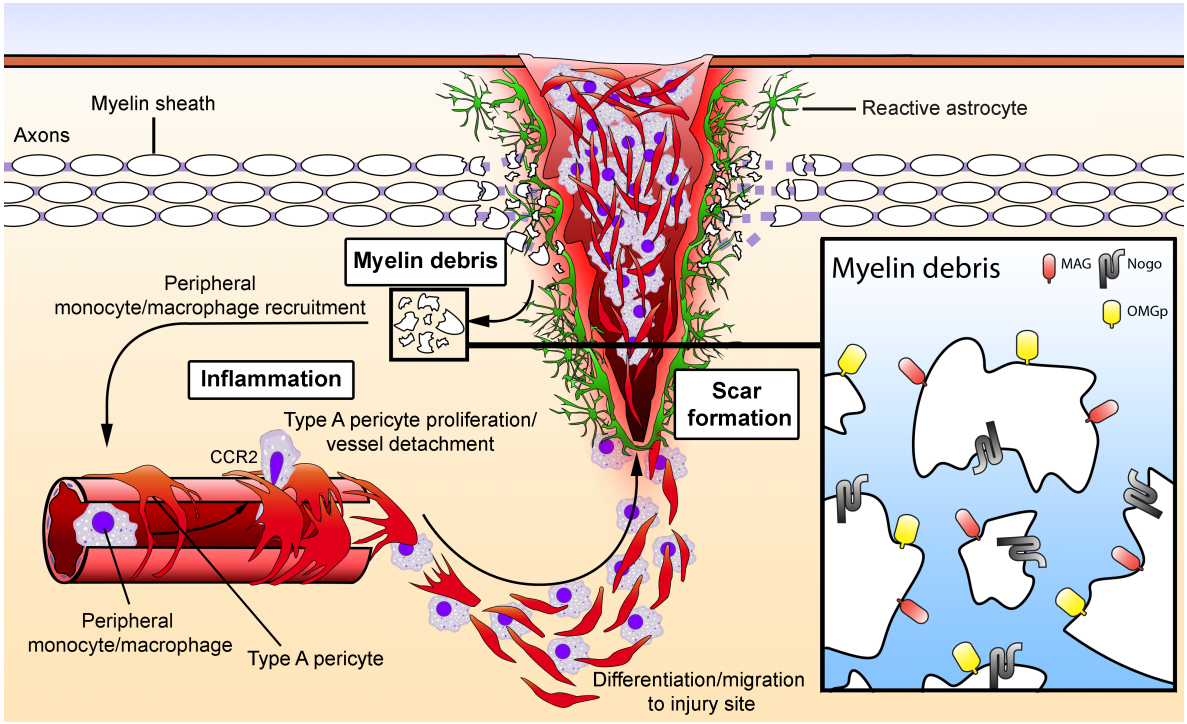

Figure 12 | The myelin-associated protein Nogo, MAG and OMgp induce fibrosis after CNS injury
Myelin damage leads to recruitment of type A pericyte-derived progeny and scar formation, a process that is dependent on the infiltration of peripherally-derived macrophages.
From (**Paper IV**). Image credits: Jannis Kalkitsas

Following SCI in humans (*140*, *367–369*) and rodents (*10*, *370*, *371*), endogenous Schwann cells, myelinating cells of the PNS, invade the lesioned spinal cord and the CNS is exposed to peripheral myelin. It remains to be tested whether fibrotic scarring is triggered by CNS exposure to PNS myelin. Similarly to the CNS, MAG and OMgp are present in PNS myelin (*372–376*) and may, in fact, recruit peripherally-derived macrophages and pericyte-derived fibrotic tissue. Additionally, it would be valuable to investigate whether proteins specific to PNS myelin, such P0 glycoprotein, the major protein component of PNS myelin (*377*), and PMP-22 glycoprotein (*378*), may also play a role in the generation of fibrotic scar tissue following SCI. MBP is also expressed by PNS myelin (*379*, *380*) but, as we have shown in the CNS, is not expected to contribute to recruitment of fibrotic tissue. Likewise, PLP, the major CNS myelin protein constituent and only marginally expressed in PNS myelin (*72*, *376*, *381*), is neither expected to cause fibrotic tissue generation, as we demonstrated in the CNS.

As revealed by our current and previous experiments (*280*), the crosstalk between microglia/macrophages and stromal fibroblasts in neuroinflammatory, demyelinating and fibrotic diseases, including MS, TBI and SCI, has become increasingly appreciated (*76*, *81*, *88*, *206*, *207*, *311*, *382*, *383*). It would be of great value to further validate how pericytes respond to an insult in the absence of an inflammatory component. In contrast to LPC-induced focal demyelination, that involves a strong inflammatory response, cuprizone-mediated systemic demyelination will enable studying pericyte-derived scarring throughout multiple brain regions and optic nerve, but sparing the spinal cord (*384*), in the context of non-inflammatory-mediated demyelination, because the BBB integrity is maintained after cuprizone treatment (*328*). Additionally, Cre-loxP-mediated conditional deletion of NgR1 (*385*) in immune cells could be employed to unequivocally verify whether pericyte-derived scarring is triggered by binding of myelin-associated proteins to NgR1 in microglia/macrophages. Likewise, additional work employing strategies that enable selective manipulation of microglia, *e.g.* Tmem119-CreER^{T2} (*386–388*) and Cx3cr1-CreER lines (*389*) crossed to mice carrying a Cre-dependent human diphtheria toxin receptor, iDTR (*390*), is needed to elucidate the role of microglia activation in pericyte recruitment and complement our current knowledge on fibrotic scar tissue formation after CNS injury (*14*, *77*, *391*).

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Response to injury is an intricate process orchestrated by multiple interacting cells types that work in consonance to reestablish tissue integrity. In the CNS, this wound healing process results in the formation of a scar, which encapsulates and prevents further spread of the damage, but simultaneously in chronic stages prevents neuroplasticity and functional recovery. The ultimate purpose of regenerative medicine is to achieve tissue repair and restoration of function without scarring. It is therefore imperative to understand the etiology of scar-forming fibroblasts, matrix-producing cells that are critically involved in the generation of scar tissue, in the context of CNS injury and disease (*392*).

This thesis demonstrates a conserved and pivotal role for a subset of pericytes as the main cellular source of stromal fibroblasts that compose fibrotic scar tissue in response to diverse CNS lesions. This group of perivascular cells, found in close association with the vasculature under homeostatic conditions, was coined type A pericytes, in order to distinguish them from other pericytes that do not exhibit scar-forming properties. Future single-cell RNA-seq and proteomic studies in combination with genetic lineage tracing, should contribute to a better understanding of different pericyte subtypes and their contribution to fibrosis. We have collected evidence that pericyte-derived scarring plays a key role in wound healing and the restoration of tissue integrity after acute tissue injury. As recognized for scar-forming astrocytes (*43*, *90*, *146*, *393*), activated microglia and infiltrating monocyte-derived macrophages (*78*, *115*, *116*, *337*, *394–399*), pericyte-derived cells engage in seemingly dual opposing roles: on the one hand they are required to seal off the injury site, as lesions failed to close when pericyte-derived scarring was nearly completely abolished; on the other hand, pericyte-derived scar tissue is not cleared off and contributes to axon regeneration failure and impaired functional recovery after CNS injury. We highlight that fibrotic scar tissue is amenable to manipulation, as moderate reduction of pericyte-derived scarring improves axon regeneration and functional recovery after SCI. We also demonstrate that fibroblast-lineage cells are present in non-neural, fibrotic scar tissue in human pathology, indicating the therapeutic relevance of the studies. Interestingly, myelin damage and myelin-associated proteins are able to trigger recruitment of scar-forming pericytes and CNS scar formation, a process that is dependent on the infiltration of peripherally-derived macrophages. Deletion of three well-established axon growth inhibitory myelin-associated proteins reduces fibrotic scarring, highlighting the crosstalk between myelin debris, inflammatory cells and fibrotic scar tissue generation by pericyte-derived cells. Therapeutic agents blocking the interaction of NgR1 with its ligands (*362–366*) or targeting Nogo-A signaling (*353–361*) may attenuate the inhibitory role that myelin-associated proteins exert on axon growth and promote functional recovery via multiple mechanisms, including reduction of pericyte-derived scarring. The work presented in this thesis demonstrates that manipulation

of inhibitory cues associated with scar tissue, namely attenuation of type A pericyte-derived scarring, has potential to improve axonal regeneration and functional recovery following SCI. However, it is insufficient to allow the majority of injured CNS axons to regenerate, suggesting that a combinatorial therapy might have a higher chance to improve axon regeneration and synaptic integration of CNS injured axons. Although some studies suggest that modifying the lesion environment has positive repercusions in supraspinal neurons by upregulating the expression of axon outgrowth, cell protection, and neural development genes (*303–306*), it is likely that activating intrinsic neuronal growth programs by exogenous manipulation (*106*, *107*) would have a stronger effect on promoting axon regeneration. Therefore, a suggested combinatorial therapy would involve activating intrinsic neuronal growth programs, attenuation of pericyte-derived scarring and intensive rehabilitation training, to increase activity-dependent synapse formation into meaningful targets and restoration of function (*400–402*).

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